Host mechanisms controlling bacterial colonization in the sea anemone *Nematostella vectensis*

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For my mom, dad and brother

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Abbreviations

°C	degrees Celsius
‰	per mille
Atf3	Activating transcriptional factor 3
AMP	Antimicrobial Peptide
AMPK	AMP-activated Protein Kinase
AP-1	Activator Protein 1
ASV	Amplicon Sequence Variant
BCG	Bacillus Calmette-Guérin
bp	base pairs
Cas9	CRISPR-associated protein 9
$CD3^+$, $CD4^+$, $CD8^+$	Cluster of Differentiation 3^+, 4^+, 8^+
CFU	Colony Forming Unit
cJUN	cellular JUN
cm	centimeter
CRE	cAMP Response Element
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleotide triphosphate
dpf	days post fertilization
dpr	days post recolonization
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
E. coli	Escherichia coli
et al.	lat.: et alii, engl.: and others
FOS	derived from the virus name FBJ (from the original researches Finkel, Biskis, and Jinkins) Osteosarcoma
g	gram
gDNA	genomic DNA
GBX	Gastrualtion Brian Homeobox
GF	germfree
GFP	Green Fluorescent Protein
HOX	Homeobox
HSP	Heat Shock Protein
hpf	hours post fertilization
IL-1β	Interleukin-1 beta
IMD	Immune Deficiency
JNK	c-Jun N-terminal Kinase
JUN	derived from the virus name avian sarcoma virus 17 (jap.: “ju-nana” (“seventeen”))
ko	knock out

L	liter
LB	Luria Bertani
Log2FC	Log 2-fold change
LPS	Lipopolysaccharide
M	molar
MAMP	Microbiome-associated Molecular Pattern
MB	Marine Bouillon
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mm^2	square millimeters
mM	millimolar
mpf	month post fertilization
mTor	mechanistic Target of Rapamycin
MAPK	Mitogen-Activated Protein Kinases
MyD88	Myeloid Differentiation factor 88
N	number of replicates
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like Receptors
NOD	Nucleotide-binding Oligomerization Domain
nm	nanometer
NM	Nematostella Medium
NK cell	Natural Killer cell
µL	microliter
µM	micromolar
µm	micrometer
OD	Optical Density
PAMP	Pathogen-associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCoA	Principle Coordinates Analysis
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pH	Potential of Hydrogen
PRR	Pattern-Recognition Receptor
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
ppt	part per thousand
rpm	rounds per minute
rRNA	ribosomal RNA
RT	Room Temperature
s	second

SEM	Scanning Electron Microscopy
sgRNA	single guide RNA
shRNA	short hairpin RNA
sp	species
TAE	Tris-Acetate-EDTA buffer
TALEN	Transcription Activator-like Effector Nuclease
TGF-β	Transforming Growth Factor beta
TIR	Toll/Interleukin-1 Receptor
TLR	Toll-like Receptor
Tm	melting Temperature
TNF	Tumor Necrosis Factor
TRE	TPA Response Element
U	unit
UV	Ultraviolet Light
Wnt	Wg (Wingless, gene first identified in *Drosophila*) + Integration site (from the Int-1 gene found as a proto-oncogene in mice)
WT	wildtype

Abstract

Animals exist as dynamic ecosystems, shaped by intricate partnerships between hosts and their microbial communities. This thesis investigates the host mechanisms that control bacterial colonization in the early-diverging sea anemone *Nematostella vectensis*, uncovering how innate immune structures, metabolic cues, and transcriptional regulators orchestrate microbial regulation in the absence of adaptive immunity. In the first chapter, I demonstrate that host nutritional status profoundly influences microbial community structure. Starvation leads to a significant reduction in microbial diversity and the selection for stress-tolerant bacterial taxa, whereas continuous feeding promotes microbial proliferation and compositional flexibility. Notably, feeding with germfree *Artemia* larvae resulted in a strong increase in bacterial abundance, suggesting that *Nematostella* actively stimulates microbial proliferation through host-mediated environmental changes rather than passive microbial introduction. In the second chapter, I identify nematosomes—motile multicellular structures in the gastric cavity—as key players in bacterial degradation. Nematosomes selectively phagocytose foreign bacteria while sparing native colonizers, revealing an unexpected level of innate immune specificity. The transcription factor cJUN emerges as a critical regulator of nematosome proliferation and function. CRISPR/Cas9-mediated knockout of *cJUN* impaired lysosomal activation during phagocytosis and led to dysbiosis, with altered microbial community composition dominated by non-native strains. These findings highlight cJUN as a central node linking immune recognition, phagocytosis, and microbial homeostasis. In the third chapter, I show that nematosomes exhibit memory-like immune behavior after microbial exposure. Nematosomes previously colonized by specific bacterial strains displayed reduced phagocytic and lysosomal responses upon re-encounter with the same strain but maintained full responsiveness to heterologous strains. This strain-specific modulation, abolished in *cJUN*-deficient polyps, resembles trained immunity—a phenomenon where innate immune cells adapt functionally based on prior microbial encounters. These results suggest that mechanisms of immune training and tolerance are deeply rooted in early metazoan evolution. Together, this work establishes *Nematostella vectensis* as a powerful model for studying the evolution of innate immune plasticity and host-microbe regulation. It reveals that even basal animals employ complex immune strategies to sculpt their microbial communities—strategies that integrate environmental sensing, selective phagocytosis, and transcriptional memory. By uncovering ancient immune mechanisms of bacterial control, this thesis reframes innate immunity not as a static, nonspecific defense, but as a dynamic, adaptive interface between host and microbiota, honed by millions of years of co-evolution.

General Introduction

The Metaorganism Concept

Marine organisms, together with their symbiotic microbiota, constitute a complex biological entity referred to as a metaorganism. This entity is defined by the dynamic interactions between the host, its immune system, nutritional status, and associated microbiota, ensuring homeostasis and overall organismal health. The metaorganism concept has fundamentally reshaped our understanding of biological interactions, acknowledging the profound influence of microbial communities on host physiology, immune functionality, and ecological adaptability.

The concept of the metaorganism describes a macroscopic host and its associated microorganisms as an integrated biological unit, emphasizing a symbiotic coexistence essential for host vitality and adaptability (**Figure-I 1**). This comprehensive perspective acknowledges that host health, physiology, and evolution cannot be fully understood without considering the microbial partners. Microbial communities associated with hosts encompass bacteria, archaea, fungi, viruses, and unicellular eukaryotes, each contributing uniquely to the functional integrity of the metaorganism. Expanding on this, recent studies have shown that specific microbial metabolites—such as short-chain fatty acids, indoles, and secondary bile acids—can influence host gene expression, epigenetic regulation, and even developmental timing, illustrating a direct biochemical interface between host and symbiont (1, 2, 3, 4, 5, 6).

Importantly, the evolutionary roots of these associations trace back to early-diverging animals such as cnidarians and sponges, indicating that host-microbiome partnerships are ancient and deeply embedded in metazoan biology. Fossil evidence and comparative genomics suggest that interactions between hosts and microbial symbionts likely emerged prior to the rise of complex organ systems, serving as foundational components of metazoan biology (7, 8, 9). In sponges, for example, diverse microbial consortia are integral to nutrient cycling and chemical defense, forming stable, heritable communities that resemble modern metaorganisms (10, 11).

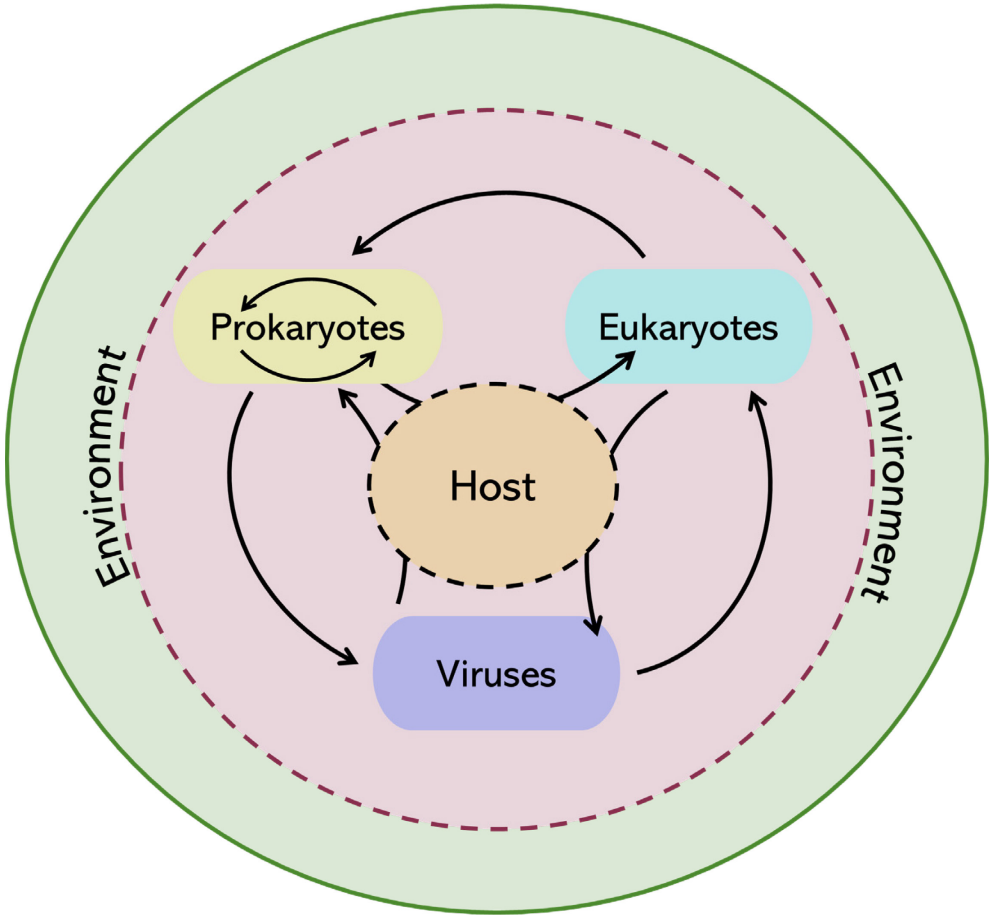

Figure-I 1 Conceptional representation of the metaorganism framework*. This diagram illustrates the metaorganism concept, emphasizing the interconnected relationships between host and its associated microscopic communities, including prokaryotes, eukaryotes, and viruses. The host (center) resides within an environmental context (outermost green cycle) that influences and is influenced by microbial interactions. Associated prokaryotes (yellow) and eukaryotic microbes (blue) engage in symbiotic and dynamic interactions with both host and each other. Viruses (purple) play a regulatory role by modulating microbial populations and impacting host fitness. The dense network of arrows underscores the continuous exchange of signals, metabolites, and genetic material that sustain homeostasis within the metaorganism (adapted and modified from Bosch et al (12)).*

Similarly, in cnidarians like hydra and corals, host-derived antimicrobials and immune signaling pathways shape highly specific microbial communities, highlighting the co-evolution of innate immunity and symbiosis (13, 14).

These early systems reveal that microbial management through immune recognition, tolerance, and selective association is not a recent innovation but a deeply conserved trait across animal lineages. For instance, microbial communities significantly influence nutrient uptake, aiding in the digestion and metabolism of dietary substances that hosts

cannot efficiently process alone. Termites and ruminants provide classic examples where symbiotic microbes facilitate the digestion of cellulose and other complex polysaccharides through specialized enzymatic systems, allowing their hosts to thrive on otherwise indigestible plant material (15, 16). Similarly, in humans, gut microbes are essential in fermenting dietary fibers and producing short-chain fatty acids, which have critical roles in metabolic regulation and immune function (17, 18). Microbes are also pivotal in modulating host immunity, impacting both innate and adaptive immune systems. They interact with host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), to initiate and regulate immune responses. Studies in mice have shown that germfree animals exhibit impaired development of immune organs and altered immune responses, underscoring the crucial role of microbial stimulation in shaping normal immune functionality (19, 20). Furthermore, symbiotic microbes provide direct protection against pathogens via colonization resistance, competitive exclusion, and production of antimicrobial substances, as exemplified by the protective role of *Lactobacillus species* against pathogenic Enterobacteriaceae in vertebrate intestines (21).

Advances in genomic sequencing technologies, including high-throughput metagenomics, transcriptomics, and single-cell sequencing, have profoundly expanded our understanding of microbiome complexity and function. Notably, metagenomic and functional gene profiling have uncovered novel microbial pathways involved in xenobiotic degradation, vitamin biosynthesis, and host neurotransmitter modulation, broadening our appreciation of symbiont-derived capabilities (22, 23). These technologies have revealed dynamic microbial community responses to environmental fluctuations, host developmental stages, and dietary changes, highlighting their adaptability and integral role in host resilience and evolutionary processes (24, 25, 26, 27).

Metabolic Influences on Microbial Community Dynamics

Across the animal kingdom, nutritional status exerts a profound influence on host-microbiome interactions. Far beyond serving as a passive backdrop, nutrient availability actively shapes the ecological and functional landscape of host-associated microbial communities. Feeding provides substrates not only for the host's metabolic needs but also for microbial growth, while starvation and caloric restriction impose selective pressures

that can restructure microbial assemblages (28, 29, 30). These shifts in microbial composition are not merely reflective of changing resource landscapes; they feed back into the host, affecting digestion, immune tone, epithelial barrier function, and overall homeostasis (31, 32). The metabolic dialogue between host and microbiota is deeply intertwined, such that even transient changes in nutrient intake can lead to durable alterations in host physiology. In this context, the microbiome emerges as both a sensor and effector of host nutritional state—a concept increasingly supported by empirical data across diverse taxa. In mammals, for example, nutrient deprivation leads to a reduction in complex carbohydrate intake, driving microbial communities to shift toward taxa capable of degrading host-derived glycans such as mucins (30, 33). Mucin glycans support microbial diversity, resist disease-associated shifts, and can be used as prebiotics to mitigate microbiota perturbations (34, 35).

In parallel, host tissues respond to nutrient fluctuations through conserved nutrient-sensing pathways, such as mTOR and AMPK, which regulate cellular metabolism and coordinate immune responses accordingly (36, 37). mTOR promotes anabolic activities, including protein synthesis, glycolysis, and lipid synthesis, while AMPK activates during nutrient scarcity (38, 39). From the microbial perspective, starvation can trigger competitive restructuring within the microbiome, favoring metabolically versatile taxa and stress-tolerant specialists (40). In human oral microbiota, *Klebsiella* and *Providencia* emerge as survivors after long-term starvation for example (41). Community-level shifts are often accompanied by changes in gene expression related to nutrient acquisition, stress resistance, and interbacterial signaling (42, 43). These adjustments allow microbial consortia to dynamically respond to resource scarcity while influencing host homeostasis. Similar principles are evident in invertebrate models. In *Drosophila melanogaster*, starvation activates transcriptional programs, like Atf3 and the IMD/Relish signaling pathway, that modulate both metabolic and immune genes, altering gut homeostasis and reshaping the microbial community (44, 45). In the nematode *Caenorhabditis elegans*, dietary restriction has been shown to affect microbial colonization and stress resistance through conserved insulin-like signaling pathways, which modulate innate immune responses and lifespan (46, 47). An interesting example are also aquatic filter feeders such as sponges. Sponge-associated microbial communities contribute significantly to

nutrient transformation, with some taxa involved in nitrogen metabolism and carbon fixation (48, 49).

However, while the microbiome composition can vary spatiotemporally, core symbiont functions often persist through functional redundancy (49). These examples underscore the bidirectional nature of metabolic-immune-microbiome interactions—a regulatory triad that maintains homeostasis under fluctuating nutritional conditions. Despite this mechanistic understanding in bilaterians, relatively little is known about how early-diverging animals integrate metabolic state with microbial regulation. Comparative studies across metazoans suggest that the metabolic regulation of symbiosis is an evolutionarily ancient feature, likely predating the divergence of major animal lineages. Many core components of nutrient sensing and immune signaling—such as AMPK, mTOR, and NF-κB—are conserved from cnidarians to vertebrates, pointing to deep evolutionary roots for this metabolic-immune-microbiome axis (37, 50).

Immunity defense strategies

Immunity is fundamental to organismal survival, involving an array of sophisticated and coordinated mechanisms to detect, respond to, and memorize encounters with pathogens (51). The immune system is typically categorized into innate and adaptive components, each displaying distinct yet interconnected roles in protecting organisms from infectious threats. To further contextualize this dichotomy, studies on jawless vertebrates such as lampreys have revealed alternative forms of adaptive immunity, relying on variable lymphocyte receptors rather than immunoglobulins (52). These findings illuminate how adaptive traits may have evolved multiple times or diversified early in vertebrate history (53). This comparative insight helps underscore the diversity of molecular strategies that support antigen-specific recognition across animal lineages.

Innate immunity represents the first line of defense, characterized by rapid and broadly reactive mechanisms. It involves physical and chemical barriers, such as epithelial surfaces, mucus layers, and antimicrobial peptides (AMPs), which provide immediate defense against invading pathogens (51, 54). Cellular components of innate immunity, including macrophages, dendritic cells, and neutrophils, mediate phagocytosis and produce inflammatory cytokines to eliminate pathogens and initiate inflammation,

attracting further immune cells to sites of infection (55, 56). Evolutionarily conserved pathways, such as the Toll-like receptor (TLR) and NOD-like receptor (NLR) signaling cascades, are crucial in recognizing pathogen-associated molecular patterns (PAMPs) to trigger innate immune responses across diverse taxa, ranging from invertebrates like *Drosophila melanogaster* to vertebrates such as humans and mice (57, 58, 59, 60). Adaptive immunity, unique to vertebrates, is distinguished by highly specific antigen recognition, immunological memory, and clonal expansion of antigen-specific lymphocytes, primarily B cells and T cells (61, 62). Upon encountering a pathogen, antigen-presenting cells activate lymphocytes, leading to clonal selection and expansion, thereby generating a highly specific and potent immune response. Adaptive immunity confers long-term protection by retaining immunological memory, ensuring a rapid and robust response upon subsequent exposures to the same pathogen (51). This phenomenon forms the basis of successful vaccination strategies in humans, where antigen-specific memory cells facilitate prompt and efficient immune responses upon re-exposure (63, 64).

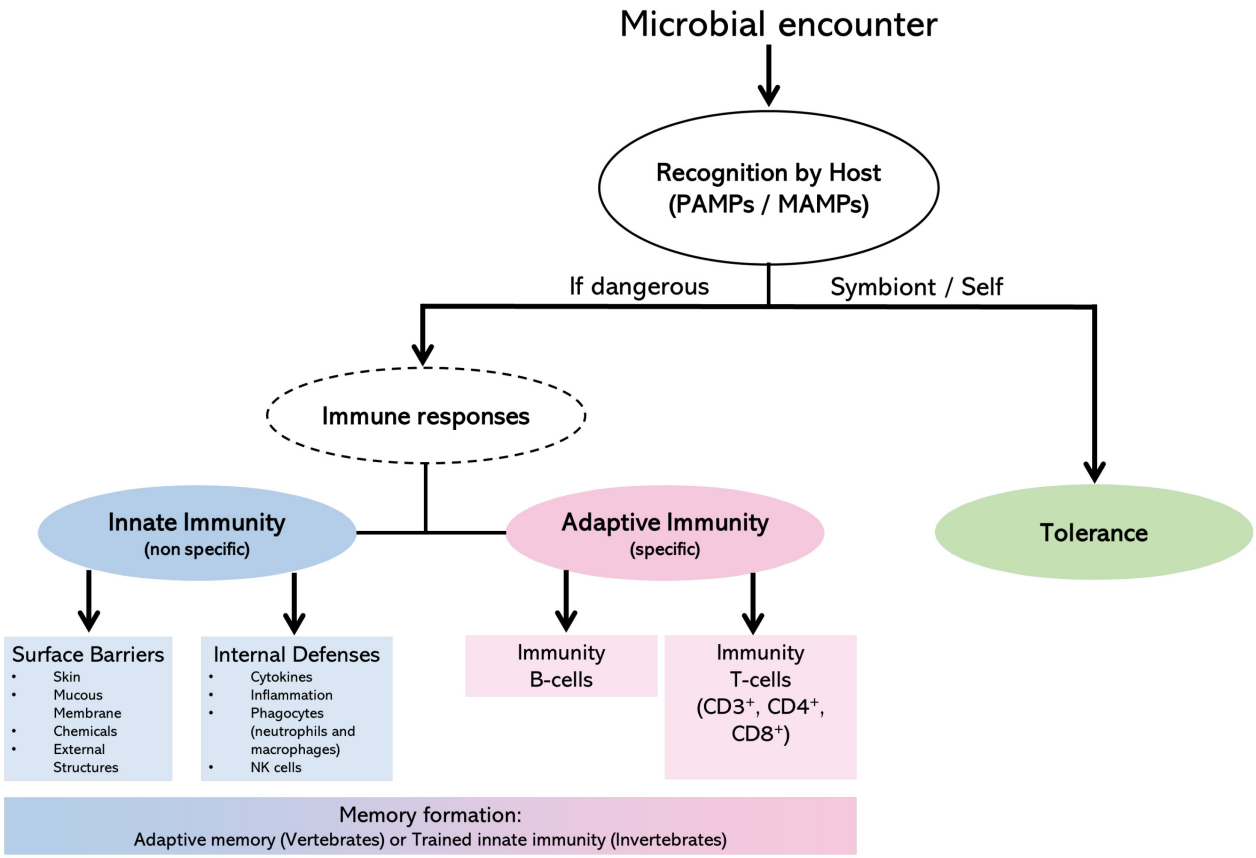

Figure-I 2 Overview of the innate and adaptive immune systems and their key cellular components. *Flowchart summarizing the immune decision-making process upon microbial encounter. Upon recognition of microbial signals (PAMPs/MAMPs), the host evaluates whether the stimulus is dangerous or associated with self or symbionts. If recognized as dangerous, immune responses are triggered, engaging both innate and adaptive immunity. Innate immunity involves non-specific defenses, including physical barriers (e.g., skin, mucus), chemical mediators, and internal responses such as inflammation, cytokine release, and phagocytosis by neutrophils and macrophages. Adaptive immunity, specific to vertebrates, engages B cells and T cells, enabling antigen-specific responses. In contrast, recognition of self or harmless microbes leads to immune tolerance, preventing unnecessary or damaging immune activation. Memory formation can occur via adaptive immunity in vertebrates or through trained innate immunity in invertebrates (51, 65).*

Historically, adaptive immunity and immunological memory were considered exclusive to vertebrates. However, recent discoveries indicate that invertebrates also exhibit adaptive-like memory features within their innate immune systems, a phenomenon termed immune priming (66, 67, 68) (**Figure-I 2**). Recent studies suggest that this phenomenon may involve epigenetic remodeling, persistent antimicrobial peptide production, and RNA-based signaling, highlighting an unexpected capacity for immune memory in organisms lacking classical adaptive systems (69, 70). Immune priming enables enhanced

responsiveness to pathogens following prior exposure, and it has been demonstrated in a variety of invertebrate taxa, including insects such as *Drosophila melanogaster* and *Bombus terrestris*, and crustaceans like shrimp (71, 72). This adaptive-like innate immune memory involves molecular mechanisms distinct from vertebrate adaptive immunity yet functionally analogous in providing enhanced protection.

In parallel to the ability of immune systems to mount stronger responses, another equally crucial feature is immune tolerance, with the capacity to limit unnecessary or self-destructive immune activity. Tolerance mechanisms are essential to prevent overactivation in response to harmless environmental stimuli, commensal microbes, or self-antigens (73, 74). In vertebrates, central and peripheral tolerance processes eliminate or inactivate autoreactive lymphocytes, involving thymic selection, regulatory T cells, and inhibitory receptor signaling (75, 76, 77, 78). Those processes are regulated by various signaling pathways, including NF-κB and calcium/NFAT signaling (79, 80). While often viewed as a uniquely vertebrate feature, tolerance mechanisms are also evident in invertebrates. For instance, in the mosquito *Aedes aegypti*, immune pathways are modulated to allow persistent viral infections without inducing pathology, suggesting that these insects actively suppress immune responses to avoid damaging host tissues (81).

This ability to balance immune activation with restraint underscores the evolutionary conservation of tolerance strategies, even in organisms lacking adaptive immunity. Together, the integration of immune priming and tolerance reveals a more nuanced view of immunity—not solely as a defense mechanism but as a dynamic system for managing interactions with both harmful and beneficial microbes. Across evolutionary time, organisms have developed diverse and sometimes convergent strategies to balance reactivity and restraint, ensuring survival not only through attack but through measured acceptance.

cJUN: A Conserved Regulator of Cellular Processes

cJUN is a member of the activator protein-1 (AP-1) family of transcription factors, a group of dimeric proteins that regulate gene expression in response to a wide range of environmental and physiological stimuli. First identified as a proto-oncogene in mammals, cJUN plays essential roles in cellular proliferation, differentiation, apoptosis, wound

healing, and immune activation (82, 83). It is one of the best-studied components of the AP-1 complex, typically forming heterodimers with other JUN (e.g., JUNB, JUND) or FOS (e.g., FOS, FOSB, FOSL1/2) family members to modulate target gene expression via binding to TRE (TPA-responsive element) or CRE (cAMP response element) motifs in gene promoters (84, 85).

One of cJUN's critical roles lies in its regulation of immune responses (83). In vertebrates, cJUN is rapidly activated via the JNK (c-Jun N-terminal kinase) signaling cascade in response to pro-inflammatory signals, pathogen-associated molecular patterns (PAMPs), and cytokine stimulation by pattern recognition receptors such as TLRs (86, 87). It is involved in regulating genes encoding pro-inflammatory cytokines (e.g., TNF-α, IL-6), chemokines, and various components of the antimicrobial machinery (88, 89, 90) (**Figure-I 3**). For instance, in macrophages, cJUN activation enhances the transcription of IL-1β and TNF, shaping the early innate immune landscape during bacterial and viral infections (91, 92). In epithelial tissues, including the gut and skin, cJUN/AP-1 signaling modulates epithelial barrier function and inflammatory responses, often in coordination with NF-κB pathways (93, 94).

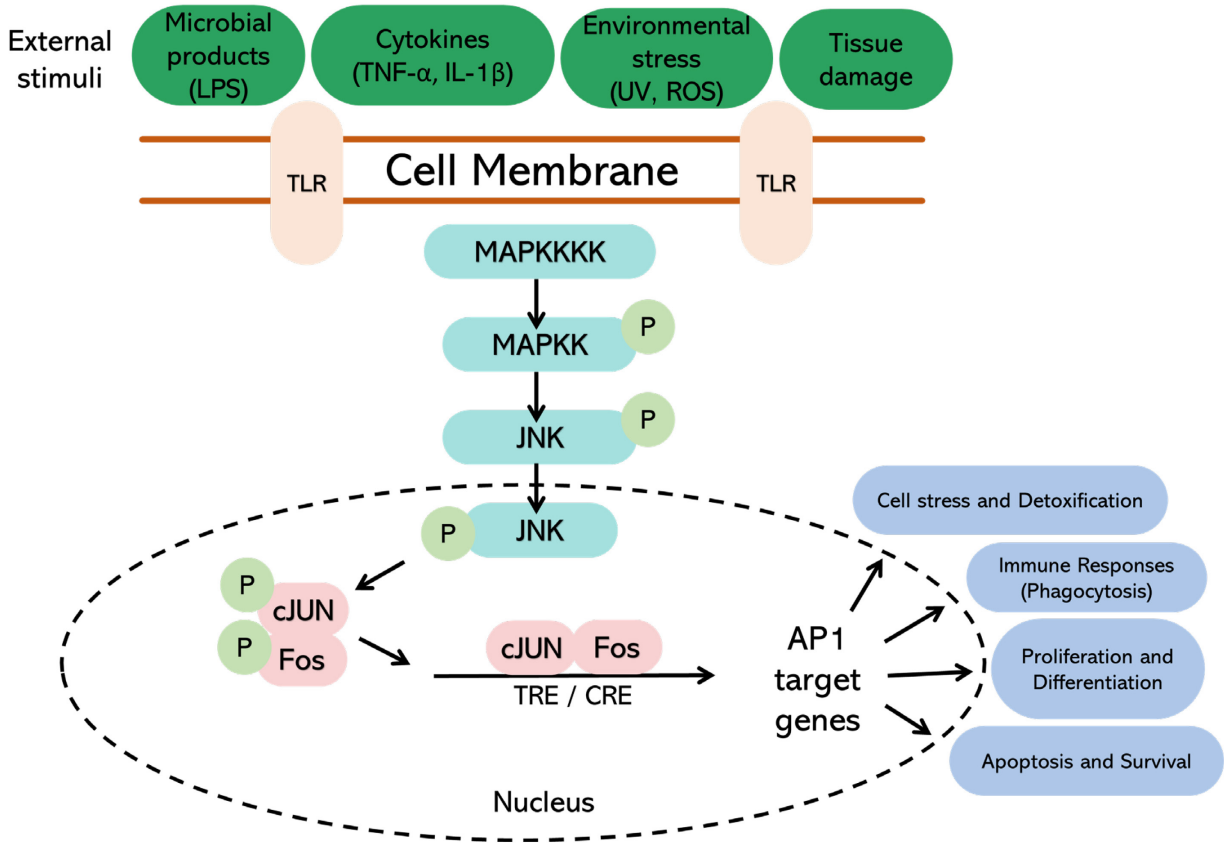

Figure-I 3 Overview of the cJUN/AP-1 signaling pathway in diverse response regulation. *External stimuli such as microbial products (e.g., LPS), pro-inflammatory cytokines (e.g., TNF-α, IL-1β), environmental stressors (e.g., UV radiation, reactive oxygen species), and tissue damage are detected by TLRs at the cell membrane. These signals activate the MAPK cascade, leading to the sequential phosphorylation of MAPKKK, MAPKK, and JNK. Activated JNK translocates into the nucleus where it phosphorylates cJUN and FOS, enabling the formation of the AP-1 transcription factor complex. The AP-1 complex binds to target DNA motifs (TRE/CRE) and drives the transcription of a broad range of genes involved in cell stress responses, immune regulation, proliferation and differentiation, and apoptosis or survival decisions. This pathway integrates external cues into adaptive transcriptional programs, allowing the cell to respond appropriately to microbial encounters, inflammatory conditions, or environmental challenges (modified from (95)) .*

In invertebrates, growing evidence points to a conserved and perhaps even ancestral role for cJUN-like proteins in immune regulation. In *Drosophila melanogaster*, JNK signaling promotes glial engulfment of neuronal debris by upregulating the phagocytotic receptor Draper expression and enhancing phagosome degradation capacity (96, 97). Also, the JNK–cJUN pathway is essential for epithelial stress responses and immune defense in the gut, acting in parallel to NF-κB signaling (98, 99). In the planarian system *Schmidtea*

mediterranea, *cJUN* is expressed in regenerating tissues and required for proper wound healing and cell fate determination (100). Even in basal metazoans such as cnidarians, orthologs of *cJUN* are expressed in stress-responsive tissues (101, 102). In addition to its role in immune activation, cJUN is involved in oxidative stress responses and cellular detoxification (103, 104). It regulates the expression of genes such as heme oxygenase-1 (HO-1) and glutathione S-transferases (GSTs), which are crucial for cellular redox balance and protection against reactive oxygen species (105, 106). In mammalian hepatocytes, cJUN is part of the cellular stress response network activated by xenobiotics and toxic insults (107). Similarly, the *Drosophila* Jun homolog Jra plays crucial role in mediating toxin responses and stress signaling (108).

Furthermore, cJUN influences cell fate decisions through its role in cell proliferation, differentiation, and apoptosis (109, 110, 111). It can act as a pro-survival factor by inducing anti-apoptotic genes such as Bcl-2, or promote apoptosis under sustained stress through upregulation of pro-apoptotic factors like Fas ligand and Bax (112, 113, 114). In mammalian stem and progenitor cells, cJUN is involved in balancing self-renewal and differentiation, often in coordination with other AP-1 family members and signaling pathways such as ERK and PI3K (115, 116).

These cross-species observations collectively indicate that cJUN is a highly conserved regulator involved in managing stress responses, immune activation, phagocytosis, and cellular adaptation. Its presence across diverse animal lineages highlights its potential role in shaping immune responsiveness and cellular homeostasis, even in organisms lacking adaptive immune systems.

Nematostella vectensis as a Model System

Building upon the conceptual framework of host-microbe symbiosis and immune system complexity, the sea anemone *Nematostella vectensis* (*N. vectensis*) has emerged as an invaluable model organism for dissecting these biological interactions. Representing an early-branching metazoan lineage, *Nematostella* offers a unique opportunity to study the evolutionary origins of immune and symbiotic mechanisms. Its simple body plan, phylogenetic positioning **(Figure-I 4B)**, and accessibility to molecular and genetic tools

make it particularly well-suited for experimental investigation into host-microbe interactions, immune regulation, and ecological adaptation.

Morphology, Life Cycle and Reproduction

Nematostella vectensis exhibits remarkable reproductive plasticity, employing both sexual and asexual reproductive modes that confer significant ecological and evolutionary advantages. Morphologically, *Nematostella* displays a radially symmetrical, elongated cylindrical body typically measuring 1–5 cm in length, with a distinct oral-aboral axis (117). The oral end features a terminal mouth surrounded by a ring of 12 to 20 tapered, contractile tentacles that are used in prey capture, mechanosensation, and chemosensation (118) (**Figure-I 4A**). These tentacles are densely packed with cnidocytes—specialized stinging cells containing nematocysts—that function in both predation and defense by discharging toxins into prey or potential threats (118). The body wall is composed of an outer epidermis and an inner gastrodermis separated by a mesoglea, an acellular gelatinous matrix. Axial patterning in *N. vectensis* involves beta-catenin, which directs gastrulation and patterns the main body axis (119, 120). A Hox-Gbx network controls radial endoderm segmentation and tentacle patterning (121). Wnt signaling plays a crucial role in oral-aboral patterning, with ectopic activation affecting ectodermal patterning along the primary axis (122). The gastrovascular cavity serves multiple functions including digestion, nutrient distribution, and waste removal (123). It is subdivided by eight mesenteries that contain gonads and house longitudinal retractor muscles, allowing for tentacle and body column retraction (124, 125). The aboral end of the animal tapers to form a pedal disc, which anchors the polyp into soft sediment using mucus and limited muscular movement.

Nematostella also displays notable tissue plasticity and regenerative capacity, supported by a relatively simple body architecture and continuous cell turnover. This makes it a tractable organism for morphological and developmental investigations, especially in studies examining epithelial patterning, cell lineage tracing, whole-body regeneration, and neuromuscular organization (126, 127, 128). Fluorescent imaging and immunohistochemistry have revealed complex nerve nets and regionalized gene expression domains, illustrating the sophistication of its neuro-muscular and digestive

systems despite its basal phylogenetic position (129, 130, 131). As such, the morphological simplicity and transparency of *Nematostella*, combined with its detailed anatomical compartmentalization, make it a powerful model for exploring conserved developmental and structural features in early-diverging animals.

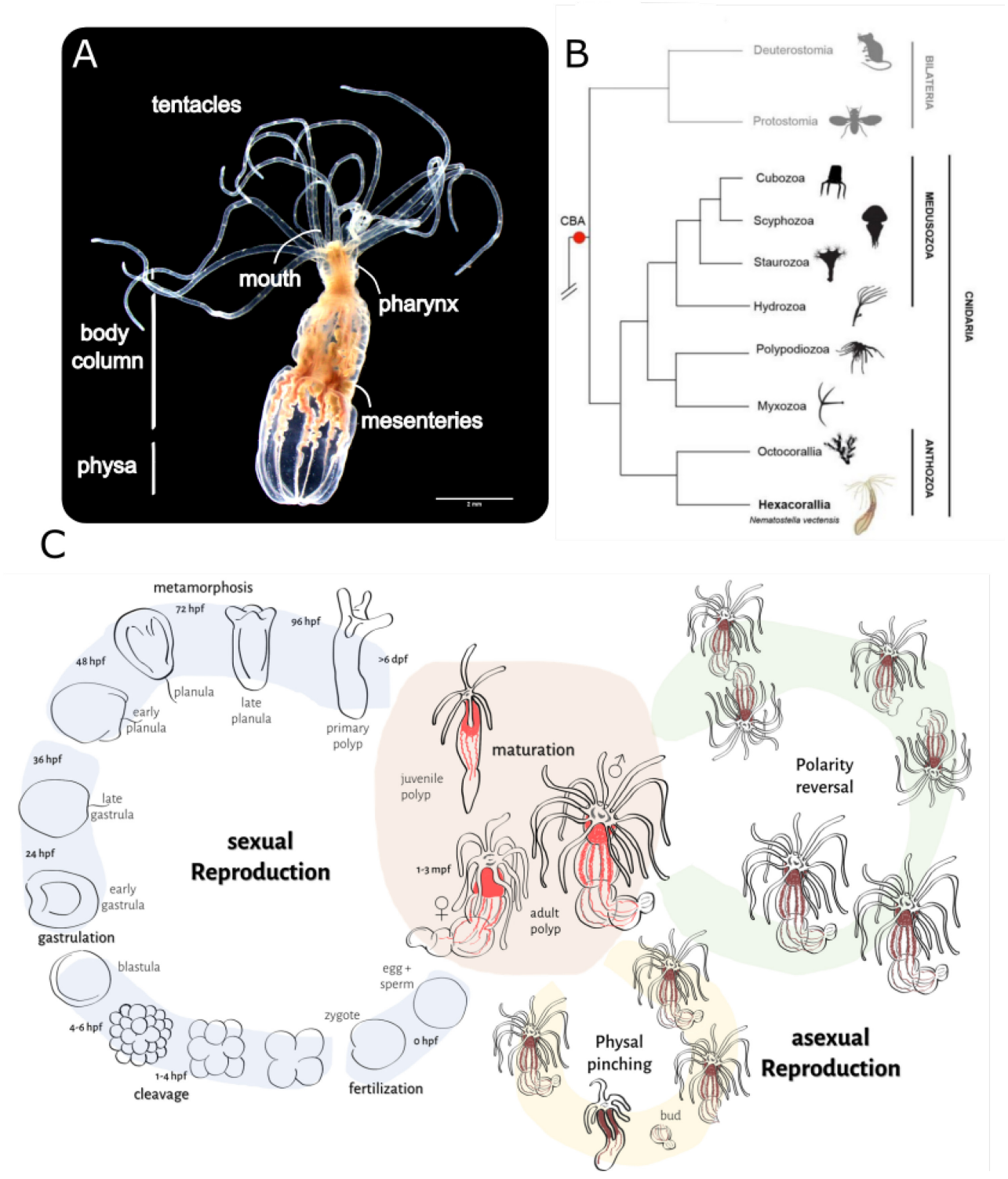

Figure-I 4 Morphology, phylogeny, and life cycle of Nematostella vectensis. ***(A)*** *Anatomical features of an adult N. vectensis polyp, showing key structures including the tentacles, mouth, pharynx, mesenteries, body column, and physa. The image highlights the radial symmetry and simple body plan of cnidarians.* ***(B)*** *Phylogenetic placement of N. vectensis within Cnidaria phylum. The tree depicts the evolutionary relationship between cnidarian lineages (e.g., Hydrozoa, Scyphozoa, Anthozoa), placing Nematostella*

within Hexacorallia as an early-branching member of Anthozoa and a sister group to the Bilateria (126). ***(C)*** *Overview of the development stages and reproductive strategies of N. vectensis. The left panel outlines the stages of sexual reproduction with embryonic development from fertilization, cleavage, gastrulation, and metamorphosis into primary polyp. The center illustrates sexual maturation from juvenile to adult polyp stages. Timepoints are indicated in hours or days post-fertilization (hpf / dpr, mpf = months post-fertilization). The right panel shows two modes of asexual reproduction observed in N. vectensis: physa pinching and polarity reversal, both resulting in clonal propagation of new individuals.*

Its sexual reproduction begins with gametogenesis, typically regulated by environmental cues such as temperature and photoperiod (125, 132, 133). Upon gamete release, external fertilization occurs in the surrounding water column, and the zygotes develop into free-swimming, ciliated planula larvae within 24 to 48 hours. These larvae possess a bilateral body axis and are capable of dispersal before settling onto a suitable substrate. Metamorphosis into sessile primary polyps is triggered by chemical cues in the environment, after which the polyps grow and differentiate into sexually mature individuals capable of gamete production.

In addition to sexual reproduction, *Nematostella* can reproduce asexually through transverse fission, whereby the body column pinches into two or more fragments, each regenerating into a complete polyp (125, 134, 135). Asexual reproduction allows for clonal propagation, population maintenance in the absence of mates, and rapid expansion in favorable habitats. This mode of reproduction is particularly advantageous in dynamic estuarine environments, enabling fast recovery from population bottlenecks and effective colonization of new or disturbed habitats. Moreover, studies have shown that the frequency of asexual fission can be influenced by environmental stressors such as salinity fluctuations, temperature or nutrient availability, highlighting a potential regulatory link between environmental sensing and reproductive strategy (134, 136).

Habitat and Ecology

Nematostella vectensis is predominantly found in estuarine habitats —transitional zones where freshwater meets marine systems—characterized by pronounced and often unpredictable fluctuations in key environmental parameters such as temperature, salinity, oxygen availability, and nutrient concentrations (137, 138). These ecosystems include shallow mudflats, tidal creeks, brackish lagoons, and salt marshes that are prone to daily

and seasonal variations due to tidal cycles, freshwater influx, and evaporation (**Figure-I 5A**). Such environments exert strong selective pressures that necessitate physiological plasticity and robust stress tolerance mechanisms (139).

Studies have shown that *Nematostella* can withstand rapid and extreme changes in salinity, tolerating ranges from hypo- to hypersaline conditions, ranging from 2 to 52 ppt. Similarly, it demonstrates broad thermal tolerance, with populations adapted to both temperate and sub-tropical climates, surviving in temperatures from -1.5°C to 32,5°C in the wild, and up to 39°C under experimental conditions, though mortality increases significantly at 41°C (117, 139, 140, 141, 142). These physiological traits make *Nematostella* a model for understanding how organisms maintain cellular homeostasis and structural integrity under environmental stress. For instance, investigations into its heat shock protein (HSP) expression profiles and osmoregulatory gene networks have provided valuable insights into stress-response systems in early-diverging metazoans (139).

Figure-I 5 Natural habitat and geographical distribution of Nematostella vectensis. (A) *Representative salt marsh habitat where Nematostella vectensis is commonly found. The species inhabits shallow brackish waters along estuarine shorelines, often burrowed within muddy or sandy sediment. Inset shows an underwater view of Nematostella polyps in situ (143).* ***(B)*** *Global distribution map of Nematostella vectensis, highlighting its native range along the eastern coast of North America (left) and introduced populations in regions such as the Isle of Wight (UK) (right) and the western United States (left). Yellow shading indicates documented coastal regions where the species has been observed (Vector graphics from Vecteezy.com).*

Geographically, populations of *Nematostella* have been reported along the Atlantic coast of North America, from Nova Scotia to South Carolina, as well as in estuarine systems of the United Kingdom and parts of the Pacific Northwest, often in isolated and genetically distinct clusters (**Figure-I 5B**) (137, 138). These populations exhibit local adaptations and

genetic differentiation, making them ideal for studying evolutionary processes such as phenotypic plasticity, genetic drift, and gene-environment interactions (27, 137, 144). Ecological research has further highlighted *Nematostella's* role in sediment bioturbation and microbial ecosystem dynamics, suggesting it may influence local biogeochemistry through its burrowing behavior and mucus secretion (145, 146).

Given its sessile lifestyle and dependence on microhabitat stability, *Nematostella* serves as a sensitive bioindicator for estuarine health and a natural laboratory for exploring resilience to environmental perturbations, including pollution, eutrophication, and climate-induced salinity shifts (143, 147).

Tools and Technologies

The genomic tractability of *Nematostella vectensis* significantly enhances its utility as a model organism across a range of biological disciplines. The organism's fully sequenced genome, approximately 450 megabases in size, exhibits a surprising degree of gene family conservation with vertebrates, especially in genes regulating innate immunity, cell signaling, and development (148, 149). This conservation makes *Nematostella* particularly valuable for evolutionary comparisons and functional studies of gene networks shared among early-diverging and bilaterian animals. Several powerful molecular tools have been developed and successfully implemented in *Nematostella*.

Short hairpin RNA (shRNA) injection and electroporation led to efficient gen knockdown during early development. This technique also has been used to investigate developmental process, including axial patterning, tissue segmentation, and tentacle formation (121, 150). More recently, CRISPR/Cas9 genome editing has been applied to generate targeted gene knockouts and knock-ins, allowing researchers to explore gene function with high precision (151). For example, in *Nematostella* CRISPR/Cas9 has been used to generate endogenously tagged proteins, enabling visualization of cellular components and dynamics (152). Beyond loss-of-function approaches, transgenic techniques have been developed to generate stable *Nematostella* lines expressing fluorescent reporter constructs under the control of tissue-specific promoters. These tools facilitate live imaging of gene expression and cell behavior, which is particularly powerful in *Nematostella* due to its transparent tissues and well-characterized embryonic stages

(153, 154). Mosaic and germline transgenesis are now being routinely used to investigate neural development, immune cell function, and host-microbe interactions at cellular resolution (**Table-I 1**).

Table-I 1 Summary of established genetic tools available for Nematostella vectensis*. The table lists commonly used methodologies for functional genomic studies in Nematostella, including genome editing, gene knockdown, and transgenesis techniques. Each tool is briefly described with indication to its function or application, and relevant references are provided to support implementation and further reading.*

Genetic Tool	Description	References
CRISPR/Cas9	Precise genome editing; Knockouts/Knock-Ins	(151)
Talens	Targeted genome editing via engineered nucleases	(151)
shRNA Knockdown	RNA interference to reduce gene expression	(150)
Morpholinos	Antisense oligos to block translation / splicing	(155, 156)
I-SecI Transgenesis	Stable transgene integration using meganuclease	(157)
Electroporation	DNA / RNA delivery via electric pulses	(150)
Microinjection	Direct injection of genetic material into embryos	(155)

Recent advances in high-throughput technologies have further expanded *N. vectensis* as a platform for integrative systems biology. Single-cell transcriptomics has revealed an unexpectedly diverse landscape of transcriptionally distinct cell types, including neuronal subtypes, secretory cells, immune-like phagocytes, and cells with context-dependent expression of innate immunity-related genes (158, 159, 160). These atlases provide a framework for understanding developmental programs, tissue architecture, and the emergence of functional specialization in early-diverging metazoans. Complementing this, bulk and spatial transcriptomics, mass spectrometry-based proteomics, and epigenomic profiling are increasingly used to analyze responses to environmental stressors and regenerative processes. In parallel, microbiome analysis tools, including 16S rRNA

sequencing and metagenomics, allow for detailed characterization of host-associated microbial communities and their shifts across tissues, developmental stages, or experimental conditions.

Together, these technologies enable a multi-dimensional understanding of how gene regulation, cell identity, and microbial signals converge to shape organismal physiology—making *Nematostella* a tractable model for addressing fundamental questions in developmental biology, immunity, and host-microbiome ecology. In addition to laboratory techniques, several online resources have made *Nematostella* a more accessible genomic model. The genome is publicly available through platforms such as Ensembl Metazoa, where users can explore annotated genes and regulatory features (161). A recently developed single-cell transcriptomic atlas further expands the utility of *Nematostella* as a reference for cell type-specific gene expression, enabling cross-study comparisons and evolutionary analyses of gene regulatory networks (158, 159, 160). These platforms collectively enhance the experimental and computational toolbox for working with *N. vectensis*, opening avenues for large-scale, integrative studies in evolutionary systems biology.

The Role of Nematosomes

Nematostella vectensis, like other cnidarians, displays a diploblastic body plan consisting of an outer ectoderm and an inner endoderm (gastrodermis), separated by a non-cellular mesoglea (162, 163). Despite this structural simplicity, *Nematostella* exhibits striking cellular diversity, with specialized cell types contributing to functions ranging from locomotion and sensation to digestion, regeneration, and immune defense.

Among the most distinctive are the cnidocytes—stinging cells that contain nematocysts capable of delivering toxins for prey capture and defense (**Figure-I 6A**) (164, 165). These cells are largely concentrated in the tentacles and continue to serve as a model for the study of cnidarian-specific innovations (101). The diffuse nerve net is composed of diverse neuronal subtypes, including sensory and peptidergic neurons, and has been shown through single-cell transcriptomics to include regionally distinct populations associated with different body zones, such as the pharynx and tentacles (129, 166).

Contractile movement is mediated by epitheliomuscular (myoepithelial) cells, which are present in both tissue layers and drive body column shortening, tentacle extension, and peristaltic behavior (167). Gland and secretory cells, particularly abundant in the pharyngeal and mesenterial tissues, secrete digestive enzymes, mucins, and antimicrobial peptides (123, 168). Transcriptomic analyses have identified subpopulations of secretory cells expressing immune effectors such as lectins and lysosomes, suggesting multifunctional roles in both digestion and microbial recognition (158, 160, 168). Phagocytic cells have been identified in the gastrovascular cavity and mesenteries and exhibit molecular hallmarks of innate immune activity, including expression of scavenger receptors, cathepsins, and lysosomal enzymes (169, 170). Though anthozoans like *Nematostella* lack interstitial stem cells (i-cells), which are prominent in hydrozoans such as Hydra (171), multipotent epithelial progenitors seems to contribute to tissue maintenance and regenerative processes throughout the animal's life. This sophisticated cellular repertoire underpins many of the core functions of *Nematostella* and sets the stage for the formation of nematosomes — unique, multicellular spheroid structures found within the gastrovascular cavity (**Figure-I 6B-D**) (170, 172).

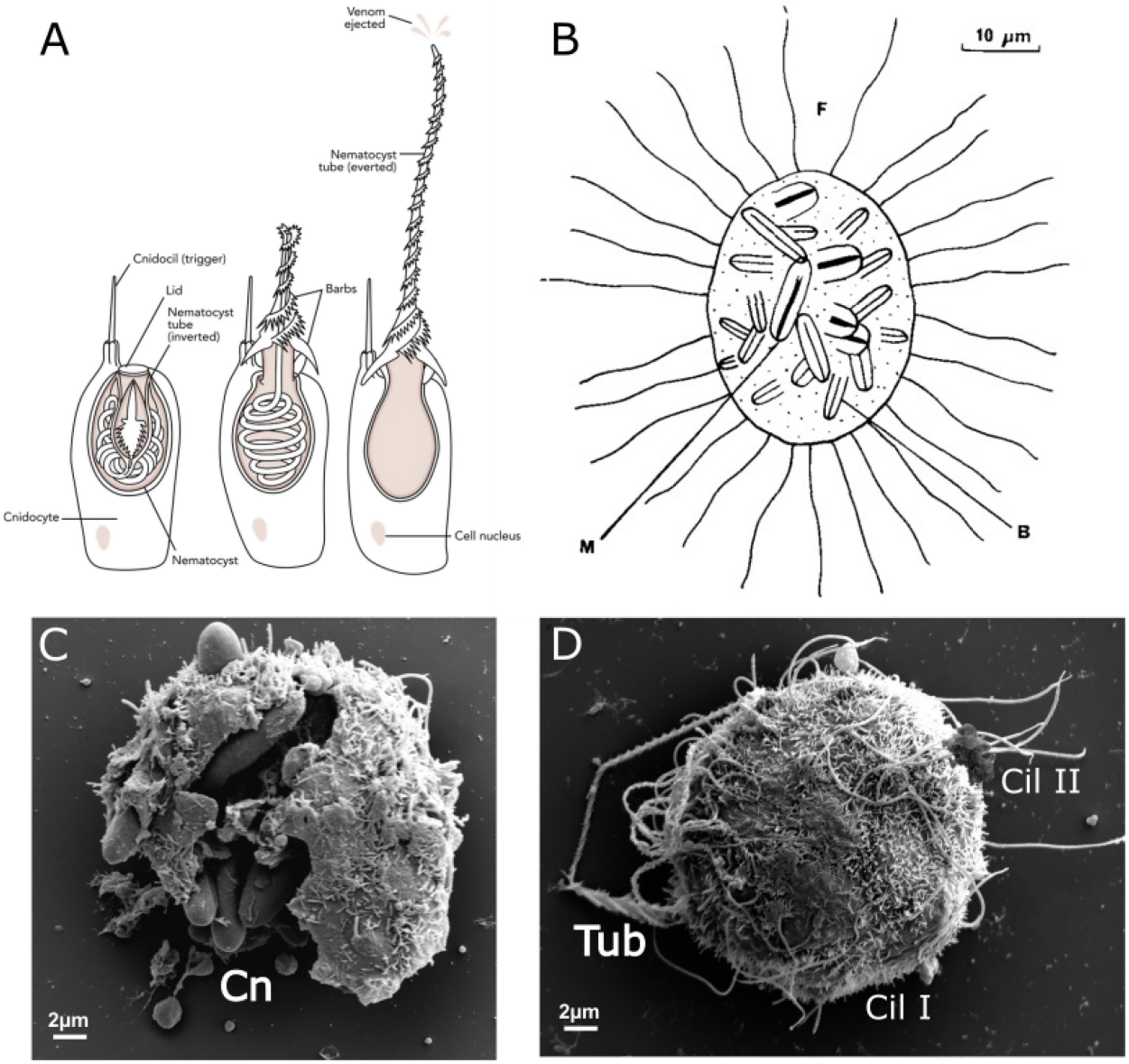

Figure-I 6 Structural and morphological features of nematosomes in Nematostella vectensis. (A) *Schematic depiction of nematocytes, the hallmark cell type found in nematosomes. Upon mechanical or chemical stimulation, the cnidocil (trigger) initiates explosive eversion of nematocyst, projecting a barbed, venom-loaded tubule used for defense and pray capture (Image by Byron Inouye (173)).* ***(B)*** *First illustration of a nematosome showing its characteristic spherical morphology, covered by motile cilia and composed of multiple embedded cell types, including cnidocytes. F = Flagella, M = Microbasic p-mastigophores, B = Basitrichs (172).* ***(C)*** *Scanning electron microscopy (SEM) image of a mature nematosome highlighting its internal composition mainly of Cnidocytes (Cn).* ***(D)*** *SEM image of a nematosome illustrating the dense network of cilia (types I (Cil I) and II (Cil II)) and cnidocyte with discharged tubule (Tub).*

These cnidocyte-rich structures emerge from the mesenterial epithelium and incorporate diverse cell types, including cnidocytes, phagocytic cells, and mucus-secreting secretory

cells (170). The presence of motile cilia on their surface enables autonomous movement within the gastric cavity, while the internal cellular organization reflects functional compartmentalization suited for pathogen detection (170). In addition, nematosomes are also incorporated into the gelatinous matrix surrounding egg packages during oogenesis. This positioning suggests a protective function during early development, potentially shielding embryos from threats (170).

As such, nematosomes represent a spatially discrete immune compartment capable of both defensive and symbiotic modulation. This mirrors immune-microbiome interfaces seen in other metazoans. For example, in insects, hemocytes phagocytose microbes and regulate gut flora via immune signaling (174). In cephalopods, like the Hawaiian bobtail squid *Euprymna scolopes*, hemocytes participate in selecting and tolerating the bioluminescent symbiont *Vibrio fischeri* within the light organ (175, 176). In vertebrates, gut-associated lymphoid tissues (GALT) like Peyer's patches orchestrate microbial sampling, tolerance, and clearance (177, 178), while even early chordates like amphioxus exhibit mucosal immune cell aggregations (179). Taken together, these systems highlight a widespread evolutionary strategy: the development of compartmentalized, immune-active structures that interface with the microbiome. Nematosomes may thus exemplify a primitive yet effective solution for microbial monitoring and immune response in early-diverging metazoans, foreshadowing more complex lymphoid architectures in bilaterians. Their dual role in immune surveillance and cnidocyte-based defense positions them as a critical interface for maintaining host-microbe equilibrium in *N. vectensis*.

Microbiome Dynamics and Host Regulation in *Nematostella vectensis*

As outlined in the preceding sections, *Nematostella vectensis* offers a unique opportunity to explore the cellular and molecular basis of host-microbe interactions. Positioned at the base of metazoan evolution, this estuarine cnidarian provides both phylogenetic context and technical accessibility, allowing researchers to investigate ancient strategies of microbial management (143). The foundational concepts of the metaorganism, innate immune specificity, and the availability of powerful genomic, transcriptomics, and spatial profiling tools, converge in *Nematostella* to enable a systems-level understanding of how animal hosts control and integrate their microbial partners and live in symbiosis.

Recent studies have revealed that *Nematostella* organizes its microbiota in a spatially structured and tissue-specific manner (27, 180). Using high-resolution 16S rRNA sequencing and imaging-based approaches, researchers have demonstrated that bacterial communities vary significantly between anatomical regions—such as the pharynx, tentacles, mesenteries, and body column—reflecting localized immune activity, epithelial secretions, and exposure gradients. The pharyngeal microbiome, for example, often contains transient food-associated bacteria, while the tentacles exhibit microbial profiles shaped by direct environmental contact and cnidocyte activity (180). This anatomical zoning of microbial niches mirrors spatial immune regulation found in vertebrate systems, such as the gut mucosa and skin, where immune activity and microbial composition are regionally tuned to balance defense and symbiosis.

One of the most compelling features of *Nematostella's* metaorganismal biology is the plasticity of its microbiome. Inhabiting fluctuating coastal environments, *Nematostella* hosts a microbial community that responds dynamically to temperature and salinity (27, 181). Experimental evidence shows that animals acclimated to elevated temperatures assemble thermally adapted microbiota, which contribute to host thermotolerance and can even confer stress resistance when transplanted into naïve individuals. Additionally, this microbiota-mediated thermal adaptation is vertically transmissible, suggesting a potential for transgenerational environmental tuning via microbial inheritance (140, 182). Importantly, these shifts are not purely environmentally driven. *Nematostella* exerts active control over microbial colonization, especially during early developmental windows (183). When germfree polyps were recolonized with microbiota from different developmental stages, the resulting communities consistently followed an ontogenetic pattern—initially resembling larval microbiota, followed by a shift toward juvenile and adult profiles. This temporal succession occurred independently of the inoculum's origin, suggesting that early colonization is host-directed, while later succession is governed by bacteria-bacteria metabolic interactions, including nitrate, sulfur, and chitin cycling. Transcriptomic data revealed that *Nematostella* upregulates chitin synthase early in recolonization, while early-colonizing bacteria exhibit enhanced chitin-degrading potential, pointing to a chitin-mediated host-microbe interface. Furthermore, mono-association assays showed that early colonizers recolonize polyps more efficiently than late colonizers, providing

functional evidence for selective host filtering during initial microbiome assembly (183) (**Figure-I 7**).

Antimicrobial peptides, mucus secretion, and pattern recognition receptors likely shape this selectivity, mirroring patterns observed in other cnidarians like *Hydra* and invertebrate models such as the Hawaiian bobtail squid (184, 185). High-throughput transcriptomic studies have identified upregulation of immune-related genes, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and components of the NF-κB and MAPK signaling pathways, during critical colonization periods, suggesting dynamic host responsiveness to microbial cues (186). A pivotal study by Brennan et al. (2017) demonstrated that *Nematostella* possesses a single functional TLR (Nv-TLR) capable of activating canonical NF-κB signaling in response to bacterial flagellin and heat-inactivated *Vibrio coralliilyticus*, a pathogenic coral-associated bacterium (186). This receptor, expressed in specific cnidocytes and nematosomes, interacts with conserved TLR adapters such as MAL and MYD88, indicating that components of vertebrate-like immune signaling are already functional in basal metazoans. Furthermore, Nv-TLR appears to play dual roles—functioning in both immune recognition and early development. Morpholino-based knockdown of Nv-TLR impairs embryonic development, suggesting a conserved developmental role, while immune assays reveal its involvement in microbial recognition and immune response. These findings offer a detailed mechanistic basis for how cnidarian innate immunity integrates microbial sensing and transcriptional activation. These gene expression shifts include increased transcription of antimicrobial peptides, such as Nv-AMP1, and lectins known to mediate microbe-host interactions. Importantly, temporal profiling indicates that these genes are transiently elevated during early recolonization and larval development, highlighting a defined window of host regulation that coincides with microbiome assembly (186). These findings underscore the role of the host as a gatekeeper that curates microbial identity from the outset of symbiosis, leveraging a toolkit of evolutionarily conserved innate immune mechanisms and restricted gene expression programs.

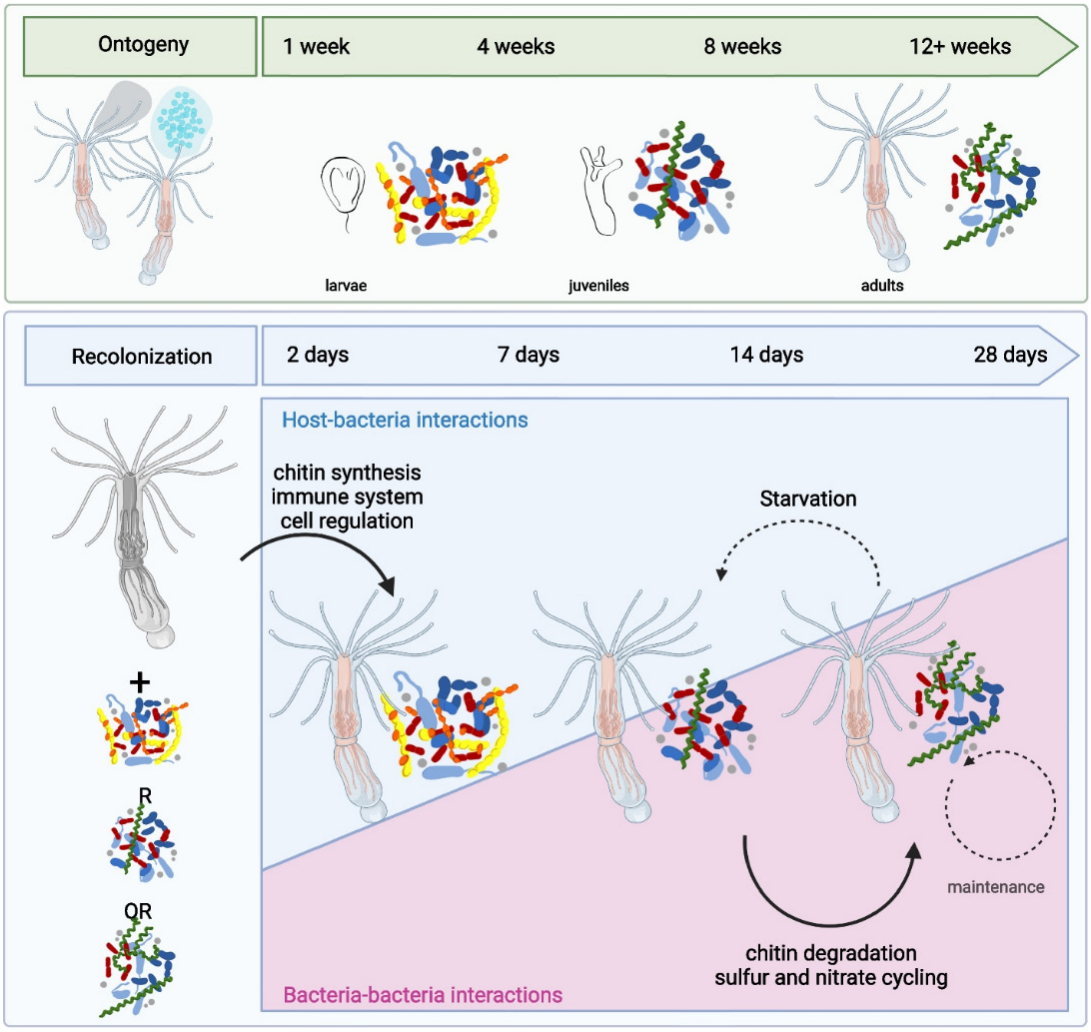

Figure-I 7 Ontogeny and temporal dynamics of microbial community interactions in Nematostella vectensis. *The upper panel illustrates the developmental stages of N. vectensis from larval (1 week) to adult forms (12+ weeks), highlighting changes in body structure and associated microbial communities (depicted as multicolored bacterial consortia). The lower panel shows microbial dynamics during recolonization over time (2–28 days) following microbial reintroduction. The gradient illustrates a shift from host-driven interactions (blue, upper left) to increasingly bacteria-driven interactions (pink, lower right). Early phases are characterized by host regulation including chitin synthesis, immune activity, and cellular remodeling. Over time, microbial community interactions begin to dominate, involving functional processes such as chitin degradation, and sulfur and nitrate cycling. Starvation alters this balance, potentially reducing microbial diversity and function toward a maintenance phase (183).*

Beyond environmental and anatomical factors, host genetic background plays a crucial role in shaping microbial community composition in *Nematostella vectensis* (181). A recent study has shown that both genotype and temperature exert significant influence on microbiota structure, with temperature emerging as the dominant factor in experimental

conditions (181). Nevertheless, microbial communities still differ between host genotypes even when reared under identical conditions, suggesting that genetic variation modulates host filtering capacity. Notably, each genotype displays a different degree of microbiota flexibility in response to environmental shifts, indicating genotype-specific microbial plasticity. These findings highlight genotype-by-environment interactions as key determinants of microbial community dynamics in *Nematostella* (181). This genotype-specific microbial filtering likely arises from variation in innate immune genes, such as pattern recognition receptors, antimicrobial peptides, and mucus-associated factors, which can differentially influence bacterial adherence, survival, and exclusion.

Despite detailed knowledge about microbiota distribution, plasticity, and environmental responsiveness in *Nematostella*, the question of how the host actively controls its microbial partners at the cellular and molecular levels remains largely unresolved. How microbial signals are integrated into host developmental and immune pathways, whether host structures like phagocytic aggregates (e.g., nematosomes) contribute to active microbial management, and whether basal metazoans possess plastic, memory-like innate immune behaviors are areas of active investigation.

Overall Aims in this work

This thesis aims to unravel the mechanisms by which the early-diverging metazoan *Nematostella vectensis* controls the composition, and functional dynamics of its microbiota. Rooted in the metaorganism concept and informed by recent advances in host-microbe research, the study investigates how environmental stimuli, immune signaling pathways, and specialized structures converge to shape microbial communities. The work proceeds through three interconnected experimental arms:

1. **Starvation and host-driven microbiome restructuring**

2. **Immune regulation through cJUN and the role of nematosomes**

3. **Long-term microbial imprinting and innate immune training**

These experimental arms draw upon an integrative methodological framework combining microbial culturing, community composition analysis through 16S rRNA sequencing, CRISPR/Cas9-based genome editing on the polyp, proteomic profiling, and high-resolution imaging. This multidisciplinary approach allows for a comprehensive examination of how *Nematostella vectensis* detects, filters, and integrates microbial signals under intrinsic and extrinsic factors. In doing so, the thesis seeks to elucidate conserved principles of microbiome modulation and advance our understanding of the evolutionary origins of host-mediated microbial control.

Chapter 1

Nutrient availability leads to immediate bacterial proliferation, while nutrient depletion stabilizes the microbiome diversity in *Nematostella vectensis*

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Abstract

Nutrient availability is a key factor shaping both host morphology and microbial community dynamics in marine metaorganisms. In cnidarians, fluctuations in feeding regimes can influence growth patterns, metabolic activity and microbiome composition, yet the extent to which these changes reflect host-driven regulation or passive environmental shifts remains unclear. Here, we investigated how increased feeding (5x/week) and starvation impact microbiome dynamics and host variability in the estuarine cnidarian *Nematostella vectensis*. Over a period of 32 days we assessed changes in polyp size, microbial diversity and abundance. Our results show that starved adult polyps undergo body contraction, likely as an adaptive response to conserve energy, whereas fed polyps exhibit fluctuations in size before stabilizing, suggesting a more dynamic response to nutrient intake. Microbiome composition differed significantly between feeding conditions. Starvation led to reduced microbial diversity and more stable yet streamlined microbiome dominated by Spirochaetota, while feeding maintained a more variable microbial community enriched in Gammaproteobacteria and a higher degree of taxonomic shifts over time. Notably, bacterial colonization in polyps fed with germfree *Artemia salina* was significantly higher than in starved and fed individuals, suggesting that *Nematostella* actively regulates microbial proliferation based on feeding status. By bridging host morphology and microbial ecology, this study provides new insights into how environmental nutrient fluctuations shape cnidarian metaorganism dynamics and underscores the importance of host-mediated microbial regulation in response to diet.

Introduction

The interplay between host nutrition and microbial community composition is fundamental to the survival and ecological function of marine invertebrates (1, 2). In cnidarians, nutrient availability influences not only host morphology but also microbiome stability, shaping interactions that contribute to health, resilience and adaptation to environmental stressors (3). While much attention has been given to the effects of temperature fluctuations, biogeography and symbiotic shifts on cnidarian metaorganisms, the impact of prolonged nutrient deprivation on both host morphology and microbial communities remains poorly understood (4, 5, 6). Starvation imposes a complex physiological challenge, affecting energy balance, tissue maintenance and immune function (1, 7, 8, 9, 10). At the same time, nutrient limitation can alter microbiome dynamics, selecting for stress-tolerant or symbiotically relevant bacterial taxa (11, 12, 13)**.** Interestingly, nutrient enrichment and predation can independently alter coral microbiomes, potentially affecting host fitness and disease susceptibility (14). The extent to which microbiome shifts occur passively due to nutrient limitations or are actively regulated by the host under starvation remain an open question in marine metaorganism research.

Nematostella vectensis, a cnidarian model system native to estuarine environments, offers a unique opportunity to study how feeding and starvation influence both host morphology and microbial interactions. Estuaries are characterized by highly variable conditions, including fluctuations in salinity, temperature and nutrient availability, making them dynamic environments that require morphological plasticity for survival (15, 16). As an infaunal species, *Nematostella* naturally experiences periods of food abundance interspersed with extended intervals of starvation (17, 18). Its ability to thrive under such conditions suggests a degree of metabolic flexibility, yet the underlying mechanisms enabling resilience remain largely unexplored. *N. vectensis* reveals a strong link between nutrition and immunity. Starvation decreases immune-related gene expression and NF-kB activity, increasing susceptibility to bacterial infection (7). Feeding induces growth and cell proliferation, while starvation causes shrinkage and cell cycle arrest, with TOR signaling playing a key role (19, 20). In corals, shifts in microbial composition are often associated with environmental stress and have been linked to increased disease susceptibility and reduced resilience (13, 21). In sea anemones and hydrozoans, microbial

communities exhibit a high degree of plasticity, influenced by external factors such as nutrient input and host immune activity (22, 23, 24).

Starvation represents a particularly strong selective pressure that may drive microbiome restructuring, either through the loss of transient, food-associated microbes or through the selective retention of taxa that contribute to host metabolism (25, 26, 27, 28). If microbiome shifts under starvation lead to a stable, low-diversity microbial community, this could suggest a form of host-driven selection, where only beneficial taxa persist under resource-limited conditions. The relationship between host feeding behavior and microbial colonization also raises important questions about the extent of the host control over microbiome composition. While feeding is expected to introduce microbes from external sources, it remains unclear whether cnidarians actively regulate microbial acquisition through immune modulation or whether microbiome shifts under starvation are primarily passive response to environmental nutrient depletion. Studies in cnidaria suggest that microbial communities are shaped by antimicrobial peptides and mucus composition (29, 30, 31), highlighting a potential role for host-driven microbiome structuring. Germfree feeding approaches provide a powerful tool to disentangle these effects, allowing to assess microbial colonization dynamics in the absence of external microbial input from food source.

To investigate how nutrient deprivation affects both host morphology and microbiome composition, we designed a study using *N. vectensis* to examine animal size and microbial diversity under contrasting feeding regimens. By implementing both long-term and short-term experimental approaches, we aim to determine whether starvation leads to measurable changes, how microbial diversity shifts over time, and whether feeding state influences microbiome plasticity. The use of germfree *Artemia* feeding treatments allows us to further explore the degree of host-microbe specificity in the absence of external microbial influx. This study provides a comprehensive framework for understanding the impact of starvation on cnidarian metaorganism, with broader implications for host-microbe interactions in fluctuating environments.

Methods

Animal Cultivation and Experimental Design

Nematostella vectensis polyps were maintained individually in six-well plates containing 16‰ Nematostella Medium (NM) under standard laboratory conditions at 18°C. A total of 90 adult female clonal polyps were used, undergoing a one-month acclimation period before the start of experiments. During this period, all animals were fed *Artemia salina* two time per week. Following acclimation, polyps were assigned to different feeding regimes. The fed group continued receiving *A. salina* five times per week, while the starved group received no food for the entire experimental period. A third group, designated as germfree fed, was provided with sterile *A. salina* to assess the impact of microbial input from food source. Two experimental timelines were implemented: a 32-day experiment to analyze both polyp surface area and microbiome composition and a three-day short-term microbiome experiment. To maintain consistent water quality, food remnants were removed daily, and plates were replaced weekly.

Surface area measurements

Polyp surface area was determined from pictures taken at nine time points over the 32-day period. Imaging was performed using a stereomicroscope (Stereomicroscope, Zeiss Stemi 08) equipped with a digital camera (Nikon D850), ensuring consistent magnification and focus settings across all measurements. To minimize variability due to feeding, fed animals were photographed at least two hours after feeding to allow complete digestion. Before imaging, polyps were transferred to dishes filled with 16‰ NM and covered for 15 to 30 minutes to reduce light exposure and promote relaxation. Once fully extended, animals were imaged under dark-field microscopy, with calibration scale included in each frame to ensure measurement accuracy. Surface area measurement were conducted using Fiji software (32).

Three-day approach and germfree *Artemia* feeding

A separate three-day experiment was conducted to assess the microbiome composition under different feeding conditions, including the use of sterile *A. salina*. A total of 45 additional polyps were divided into the same feeding groups as in the long-term

experiment. Germfree *A. salina* was prepared using modified bleaching protocol (33) to ensure sterility without antibiotic interference. The sterility of the *A. salina* larvae was verified by homogenization and plating on Marine Broth (MB) agar, followed by incubation to assess microbial growth.

Microbiome analysis and CFU Quantification

Microbiome analysis was performed at selected time points using five replicates per group. To remove residual debris and non-adherent microbes, polyps were washed with sterile 16‰ NM before homogenization. Homogenized samples were serially diluted and plated onto Marine Broth (MB) agar under sterile conditions. Agar plates were incubated at room temperature for one to two days, and colony-forming units (CFUs) were manually counted. Microbial abundance per polyp was determined by applying standard dilution factor calculations.

Genomic DNA isolation and 16S rRNA Gene Sequencing

Genomic DNA (gDNA) was extracted from polyps in both the 32-day and three-day experiments, as well as from untreated and germfree *A. salina* samples, using the DNeasy® Blood & Tissue Kit (Qiagen) with protocol modifications. Proteinase K (Thermo Scientific) was used instead of the enzyme provided in the kit, and centrifugation speeds were adjusted based on laboratory equipment specifications. Sample lysis was enhanced by immediate vortexing after buffer addition, and ethanol was included to improve DNA recovery. For 16S rRNA gene sequencing, bioinformatic analyses were performed using Qiime 2 (version 2021.11) (34). Raw sequences were demultiplexed and quality-filtered using the q2-demux plugin, followed by denoising with DADA2 to generate amplicon sequence variants (ASVs) (35). Sequences were aligned using MAFFT, and phylogenetic trees were constructed using FastTree2 (36, 37). Before calculating diversity metrics, all samples were rarefied to 900 sequences per sample. Alpha diversity estimates included observed features and Faith's Phylogenetic Diversity, while beta-diversity was assessed using Bray-Curtis dissimilarity, Jaccard distance, Weighted and Unweighted UniFrac distances (38, 39, 40). Principal Coordinate Analysis (PCoA) was applied to visualize community differences. Taxonomic classification of ASVs was performed using q2-feature

classifier-sklearn naïve Bayes classifier trained on the Greengenes 13_8 99% reference dataset (41). Further statistical analysis and visualization were conducted using OriginPro (Version 2021. OriginLab Corporation, Northampton, MA, USA.).

Results

Starvation reduces polyp growth and microbial abundance over time

All polyps were initially maintained under common feeding regime of twice per week before experimental phase, in which they assigned to distinct feeding conditions: fed polyps received an increased feeding frequency (5x/week, **Figure 1-1A**), while starved polyps were completely deprived of food. Additionally, a subset of polyps was fed daily with germfree *Artemia salina* nauplii **(Figure 1-1D)** to evaluate the contribution of microbial absence in diet on host's bacterial abundance. This transition in feeding conditions allowed us to examine the effects of sustained feeding versus nutrient deprivation on polyp growth and microbial dynamics.

Polyp growth dynamics differed markedly between feeding conditions (**Figure 1-1B**). High-frequency fed polyps exhibited fluctuations in surface area, initially decreasing from approximately 77 mm^2 at day 0 to 63 mm^2 by day 3, followed by a decline around day 11 and subsequent stabilization near 45 mm^2 by day 32. This pattern suggests that while continuous nutrient intake supports polyp maintenance, short-term fluctuations occur before eventual stabilization. In contrast, starved polyps showed progressive size reduction, decreasing from 77 mm^2 at day 0 to approximately 60 mm^2 by day 8, and further shrinking to 30 mm^2 by day 32. These results indicate that starvation leads to size reduction, likely as an energy-conserving survival strategy. Statistical analysis using two-way ANOVA with repeated measures confirmed a significant effect of time on surface area ($p < 0.001$). However, neither feeding status ($p = 0.399$) nor the interaction between time and feeding ($p = 0.321$) reached statistical significance, suggesting that while both groups

exhibited temporal changes in surface area, the difference between high-frequency fed and starved polyps was not statistically robust over time.

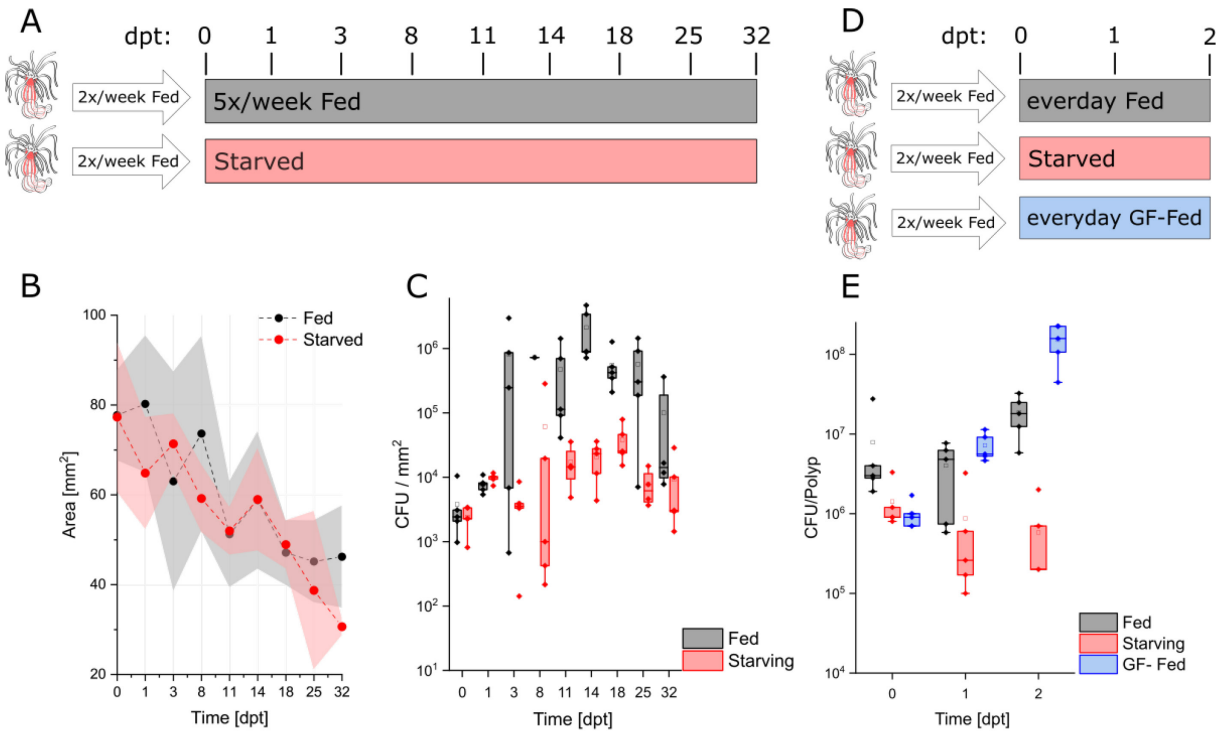

Figure 1-1 Starvation reduces polyp growth and microbial abundance. (A) Schematic representation of the experimental setup. All polyps were initially fed twice per week before being assigned to three experimental groups: continuously fed (black) and starved (red). Sampling occurred at multiple time points (0,1,3,8,11,14,18,25 and 32 days post-treatment [dpt]). **(B)** Surface area (mm^2) of fed (black) and starved (red) polyps over time. Points represent mean values; shaded area indicate standard error. Fed polyps initially increased in surface area before stabilizing, while starved polyps showed a steady decline. **(C)** Colony-forming units (CFU) per mm^2 of polyp over time in fed (grey) and starved (red) groups. Data are shown as boxplots, with individual data points overlaid. Fed polyps exhibited consistently higher microbial abundance, whereas starved polyps displayed lower CFU values. **(D)** Revised schematic showing an additional experimental condition where polyps were fed daily with germfree *Artemia* (GF-Fed, blue), alongside the previously described fed and starved conditions. **(E)** CFU per polyp over time in fed (grey), starved (red), and germfree-fed (blue) groups. Germfree-fed polyps exhibited distinct microbial profiles compared to the other groups, with consistently higher CFU values.

While changes in polyp size alone did not significantly differentiate feeding conditions, the microbial abundance profile revealed a striking contrast between fed and starved polyps **(Figure 1-1C)**. Fed polyps consistently harbored higher bacterial load than starved polyps, with microbial abundance peaking at day 14, exceeding 10^6 CFU/mm^2, before gradually declining. In contrast, starved polyps maintained a lower microbial load, with CFU levels

remaining around 10^4 CFU/mm² throughout the experiment. Two-way ANOVA revealed significant effects of feeding ($p < 0.001$), time ($p = 0.008$), and their interaction ($p = 0.01$), confirming that both feeding and time significantly influenced microbial abundance. This indicates that despite similar polyp size trajectories, feeding exerts a critical influence of microbial load.

To test whether the increase in bacteria levels after feeding was due to the proliferation of symbionts or the introduction of new bacteria through the diet, we carried out a second short-term feeding experiment in which we also fed germfree *Artemia* larvae **(Figure 1-1D)**. Interestingly, the bacterial abundance increased significantly in germfree-fed polyps **(Figure 1-1E)** suggesting that indeed bacterial proliferation is causing the increase in bacterial abundance after feeding. Germfree-fed polyps exhibited a larger increase in microbial abundance at day 2, surpassing 10^8 CFU/mm², which was significantly higher than in polyps fed with normal *Artemia* larvae. Two-way ANOVA confirmed a significant effect of condition ($p < 0.0001$), time ($p < 0.0001$), and their interaction ($p < 0.0001$), underscoring the strong influence of feeding status and time on microbial abundance. The unexpectedly high bacterial load observed in germfree-fed polyps suggests that feeding induces host-mediated changes, such as altered nutrient secretion or metabolic flux, that promote microbial proliferation beyond direct bacterial introduction through diet.

Microbial diversity declines over time with feeding modulating community restructuring

To test whether shifts in microbial diversity and community composition are linked to nutrient availability of the host, we analyzed microbial alpha and beta diversity in high-frequency fed (5x/week) and starved polyps over a 32-day period **(Figure 1-2)**. Microbial diversity, assessed through Faith's Phylogenetic Diversity (PD) and Evenness (Pielou's index), revealed distinct trends between feeding conditions **(Figure 1-2A and 1-2B)**. Faith's PD showed a significant decline over time in both conditions, with no major differences in the overall rate of decline between feeding and starving. Fed polyps retained higher levels of phylogenetic diversity overall but exhibited some fluctuations in diversity across time points. Two-way ANOVA confirmed a significant effect of time on Faith's PD

($p = 0.001$), while feeding condition alone was not statistically significant ($p = 0.131$). However, the interaction between time and feeding was significant ($p = 0.050$), indicating that the impact of starvation on diversity increased over time.

Similarly, microbial evenness significantly decreased in starved polyps, suggesting that certain microbial taxa became dominant while others declined **(Figure 1-2B)**. Two-way ANOVA revealed significant effects of time ($p = 0.002$) and feeding condition ($p < 0.0001$), indicating that fed polyps retained a more balanced microbial community structure, while starvation led to a microbiome dominated by fewer taxa.

To further understand how microbial composition changed over time, we analyzed Jaccard distances to 0dpt, which measure how different the microbial communities became relative to their initial stage **(Figure 1-2C)**. Fed and starved polyps exhibited a continuous shift in microbial composition, with Jaccard distances increasing over time at similar rates, indicating ongoing microbial restructuring in both conditions. This suggests that while microbial turnover was reduced in starved polyps, compositional changes still occurred over time, though at a slower rate. Statistical analysis confirmed significant effects of feeding condition ($p < 0.0001$), time ($p = 0.0285$), and their interaction ($p = 0.007$), highlighting that microbiome changes were strongly influenced by both feeding state and duration.

To assess the overall differences in microbial community composition, Adonis and ANOSIM analyses were performed using multiple beta diversity metrics, including Bray-Curtis, Jaccard, Weighted UniFrac and Unweighted UniFrac distances **(Table 1-1)**. These analyses confirmed significant differences in microbial composition between feeding conditions (Adonis $p < 0.001$, ANOSIM $p < 0.001$ for all metrics), supporting the distinct microbial community structures in fed and starved polyps. The strongest differences were observed in Weighted UniFrac (Adonis $R^2 = 0.238$) and Bray-Curtis (Adonis $R^2 = 0.210$), indicating that phylogenetic and abundance-based differences primarily drive the divergence between fed and starved microbiomes. Interestingly, while Jaccard distances had a lower effect size in Adonis analysis ($R^2 = 0.010$), ANOSIM results ($R = 0.344$, $p < 0.001$) indicate that presence-absence differences still play a significant role in differentiating microbial communities between feeding conditions. This supports the use of Jaccard distances in further analysis. Temporal effects were also detected, with Jaccard distances (Adonis $R^2 = 0.087$, $p < 0.001$) indicating significant shifts in community

composition over time. Importantly, while fed polyps exhibited continuous shifts in microbial composition, the absence of major loss in microbial diversity indicates that 5x/week feeding does not lead to overfeeding-induced dysbiosis but rather supports a stable and dynamic microbiome.

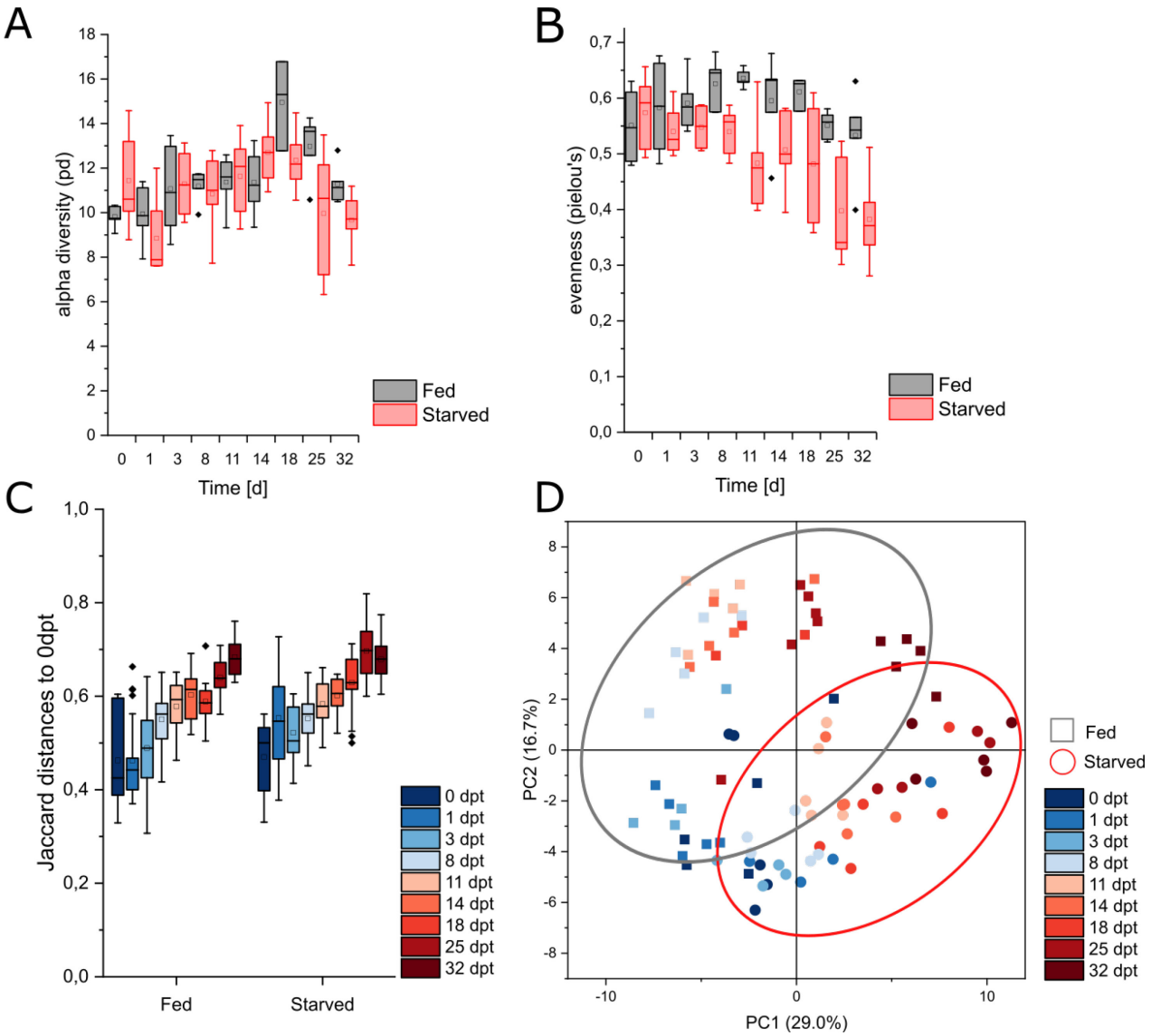

Figure 1-2 Starvation alters microbial diversity and community composition over time. **(A, B)** Alpha diversity measures in fed (grey) and starved (red) polyps over time. **(A)** Faith's phylogenetic diversity (PD) and **(B)** microbial evenness (Pielou's index) show that microbial diversity is consistently lower in starved polyps compared to fed polyps. **(C)** Jaccard distance to 0 dpt for fed and starved polyps at multiple time points. Fed polyps exhibit a gradual shift in microbial community composition, with significant differences between early and later time points. **(D)** Principle coordinate analysis (PCoA) of microbial community composition based on Jaccard distances at multiple time points (0 to 32 dpt). Fed (black) and starved (red) polyps show distinct clustering, indicating divergence in microbial community structure. The first principle

coordinate (PC1) explains 29.0% of the variation, while the second principle coordinate (PC2) accounts for 16.7% of the variation.

Table 1-1 Statistical summary of ADONIS and ANOSIM test on Bray-Curtis, Jaccard, Weighted UniFrac and Unweighted UniFrac distance matrices, comparing microbial dissimilarities between fed and starved group under the influence of the condition and dpt. R-values and associated p values, indicating significance and strength of dissimilarity between the both condition of being fed and starved.

Parameter	Metric	Adonis R^2	Adonis p	Anosim R	Anosim p
condition	Bray-Curtis	0.210	<0.001	0.357	<0.001
	Jaccard	0.010	<0.001	0.344	<0.001
	Weighted Unifrac	0.238	<0.001	0.355	<0.001
	Unweighted Unifrac	0.089	<0.001	0.274	<0.001
dpt	Bray-Curtis	0.031	0.05	0.095	0.004
	Jaccard	0.087	<0.001	0.299	<0.001
	Weighted Unifrac	0.030	0.064	0.055	0.042
	Unweighted Unifrac	0.069	<0.001	0.255	<0.001

Principal coordinate analysis (PCoA, **Figure 1-2D**) was performed using Jaccard distances, as ANOSIM results indicated strong clustering based on presence-absence differences. This metric was chosen to highlight compositional shifts over time, independent of abundance -based variations. The analysis demonstrated that microbial communities in fed and starved polyps became increasingly distinct over time. The first principle coordinate (PC1) explained 29.0% of the variation, while the second principle coordinate (PC2) accounted for 16.7%, showing a clear separation between feeding conditions. Fed and starved polyps both showed progressive changes in microbiome composition over time, with no strong indication of one condition exhibiting greater shifts than the other.

These findings reveal that starvation leads to a decline in microbial diversity and restricts community restructuring over time, while feeding maintains higher diversity and promotes continuous shifts in microbiome compositions. The progressive divergence between fed and starved microbiomes highlights the strong influence of nutrient availability on microbial community dynamics and suggests that starvation stabilizes the microbiome, potentially by limiting environmental inputs that drive microbial turnover.

Nutrient deprivation shifts microbial composition, favoring Spirochaetaceae over Vibrionaceae

Following the observed effects of feeding and starvation on microbial diversity and community structure, we next analyzed taxonomic composition to determine how specific bacterial groups respond to prolonged nutrient deprivation. The relative abundance of major bacterial taxa and differentially abundant ASVs (amplicon sequence variants) were compared between fed and starved polyps across a 32-day period **(Figure 1-3)**. The results indicate that feeding maintains a relatively stable microbial composition, while starvation promotes shifts in bacterial communities, leading to an enrichment of specific taxa such as Spirochaetota in the absence of regular nutrient inputs. The overall taxonomic composition differed notably between fed and starved polyps over time **(Figure 1-3A)**.

In fed polyps Alphaproteobacteria and Gammaproteobacteria remained the dominant bacterial classes throughout the experiment, with their relative abundance remaining stable across all time points. In contrast, starved polyps exhibited increased variability in taxonomic composition, particularly at later time points, with notable increase in the relative abundance of Firmicutes and Spirochaetota already at 8 dpt. These findings suggest that starvation leads to shifts in resource availability and microbial competition dynamics, favoring certain bacterial taxa adapted to nutrient-depleted conditions.

To further identify specific bacterial ASVs that were differentially enriched between feeding conditions, we generated a heatmap of relative abundance at the genus level **(Figure 1-3B)**. Log2 fold changes in bacterial abundance were calculated relative to 0 dpt within each feeding condition, allowing us to assess how microbial taxa shifted over time in fed and starved polyps. Several genera within Gammaproteobacteria and Alphaproteobacteria remained consistently abundant in fed polyps, including *Vibrio*, *Pseudorhodobacter*, and *Kiloniella*, suggesting that regular feeding supports the persistence of these bacterial groups. In contrast, starved polyps exhibited a marked increase in the relative abundance of Spirochaeta **(Figure 1-3B)**.

These shifts suggest that specific ASVs within these genera may be differentially selected depending on nutrient availability, potentially driven by altered host-microbe interactions or changes in competitive exclusion dynamics. The temporal trajectories of dominant taxa

reveal distinct microbial responses to nutrient deprivation. The relative abundance of a dominant Gammaproteobacteria taxon with the Vibrionaceae decreased sharply in starved polyps, whereas it remains stable in fed individuals **(Figure 1-3C)**. In contrast, a key Spirochaetaceae member followed an opposite trajectory, increasing in starved polyps over time while remaining low in fed individuals **(Figure 1-3D)**. These patterns indicate that starvation may lead to the loss of specific microbial associates while selecting for stress-tolerant taxa like *Spirochaetaceae sp*. capable of surviving in nutrient-limited environments.

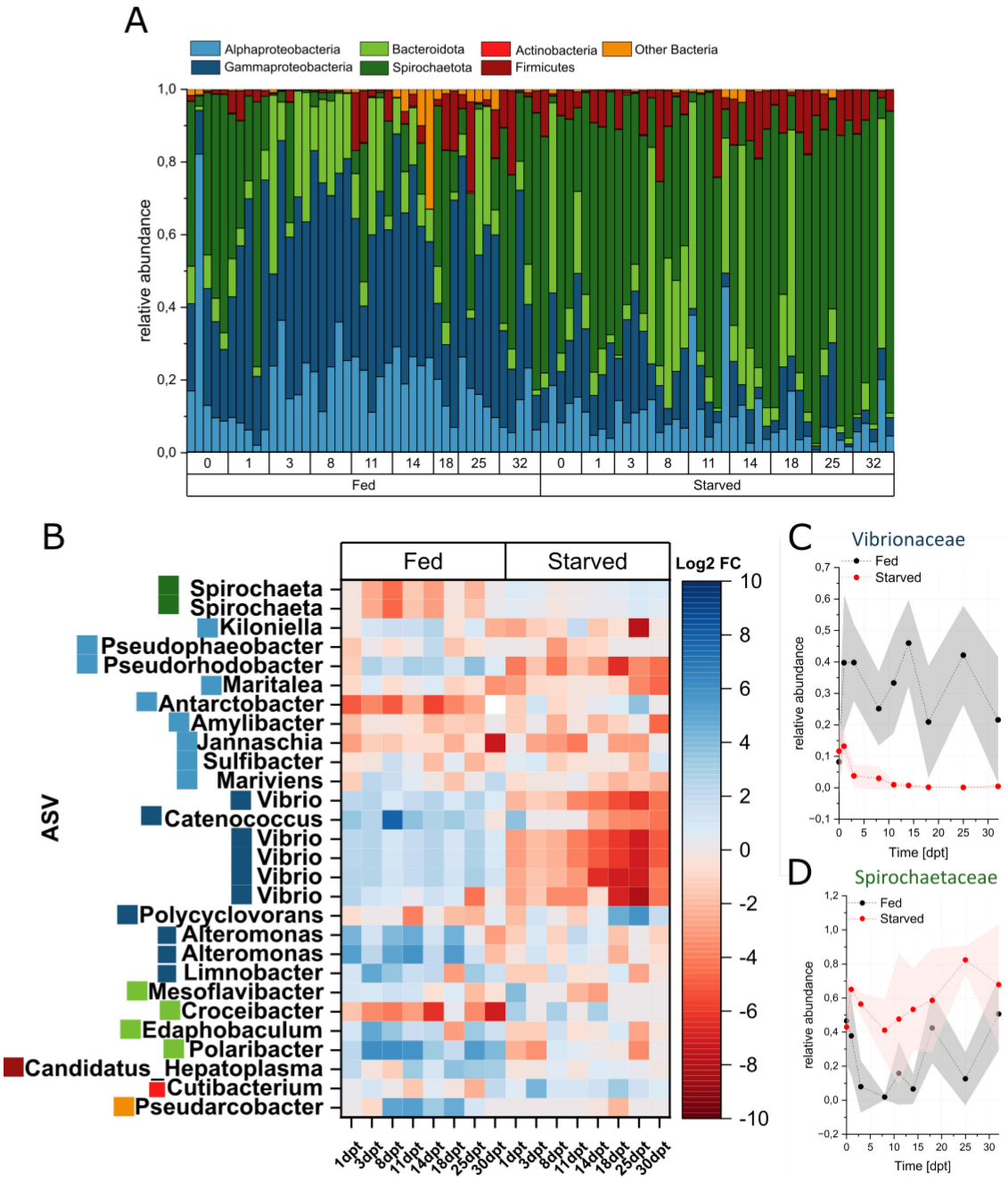

Figure 1-3 Starvation reshapes microbial taxonomic composition, increasing variability over time. **(A)** Relative abundance of bacterial taxa in fed and starved polyps over time. Stacked bar plots show relative proportions of major bacterial classes across multiple time points (0, 1, 3, 8, 11, 14, 18, 25, 32 dpt) in fed and starved polyps. Dominant bacteria classes include Alphaproteobacteria (blue), Gammaproteobacteria (dark blue), Bacteroidota (green), Spirochaetota (light green), Actinobacteria (red), Firmicutes (dark red), and other bacteria (yellow). Fed polyps display a stable microbial composition dominated by Alphaproteobacteria and Gammaproteobacteria, while starved polyps exhibited increasing variability and a higher relative abundance of Spirochaetota. **(B)** Heatmap displaying differentially abundant bacterial taxa in fed and starved polyps. Color intensity represents log2 fold changes in relative abundance, with red

indicating enrichment and blue indicating depletion. Several Spirochaetota taxa are enriched in starved polyps, while Gammaproteobacteria and Alphaproteobacteria remain dominant in fed polyps. **(C)** Relative abundance changes of Vibrionaceae in fed and starved polyps over time. Fed polyps maintain relatively stable Vibrionaceae levels, whereas starved polyps exhibited a pronounced decline in their abundance. **(D)** Relative abundance changes of Spirochaetaceae in fed and starved polyps over time. Starved polyps show a notable increase in Spirochaetaceae, whereas fed polyps maintain lower and more stable levels.

The observed taxonomic restructuring under starvation conditions aligns with the previously detected decline in microbial diversity and stabilization of community composition **(Figure 1-2)**. While regular feeding supports a dynamic and adaptive microbiome, starvation appears to favor a selected group of taxa that may be better suited to persist in resource-limited environments. Together, these results suggest that nutrient deprivation drives microbial succession, leading to long-term taxonomic shifts that differentiate the microbiomes of fed and starved polyps.

Discussion

Feeding drives rapid microbial shifts, highlighting host regulation

One of the most striking findings of this study is the immediate bacterial response to feeding, as seen in the sharp increase in microbial abundance following nutrient intake **(Figure 1-1E)**. The rapid colonization of polyps fed with germfree *Artemia* suggests that microbial growth is not solely food-derived bacteria but rather stimulated by host-mediated factors, such as increased metabolic flux. This observation indicates that feeding directly influences the microbial load, likely by altering nutrient availability and creating favorable conditions for bacterial proliferations. Similar effects have been documented in corals, where host-derived metabolites and mucus secretion act as key regulators of microbial dynamics (13, 42). These findings reinforce the idea that the host actively modulates microbiome composition in response to nutrient intake, beyond simply acquiring microbes from food sources. In cnidarians, immune signaling, antimicrobial peptides and mucus composition are known to regulate microbial recruitment (31, 43, 44).

Further evidence from research on *N. vectensis* demonstrates the host's strong influence on microbiome assembly, underscoring the point that microbiome composition is not solely dictated by environmental factors but is also actively modulated by the host. Initial colonization is shaped by host-driven selection, and microbial succession is further influenced by bacterial interactions (45). Bacterial community composition in *N. vectensis* is closely linked to host developmental stages and dynamically responds to environmental variations (6). Additionally, both host genotype and environmental factors contribute to microbiome plasticity, with genotype-environment interactions influencing microbial community structure over time (22, 46). Given its relevance to microbiome regulation, this concept aligns with the host's active role in shaping microbial diversity based on nutrient availability, as described earlier. These insights further support the findings on microbial proliferation in response to feeding and stabilization under starvation, highlighting the host's regulatory influence in microbial dynamics.

Starvation reshapes microbiome composition and reduces diversity

Nutrient deprivation imposes strong metabolic constraints on cnidarians, forcing trade-offs between survival and growth. Starvation not only significantly impacts cnidarian

morphology, but also leads to downregulation of genes involved in metabolism, cellular respiration and immunity in *N. vectensis* (7, 47). During starvation, juvenile *N. vectensis* exhibits exponential shrinkage rates and dramatic cell loss, with >7% of cells shifting between cell cycle phases (19). This metabolic plasticity is driven by muscular-hydraulic machinery, which influences body size and shape through cavity inflation and muscle organization (48). Adult *N. vectensis* polyps undergoing prolonged starvation exhibited gradual size fluctuations rather than continuous contraction **(Figure 1-1B)**, suggesting an adaptive strategy balancing energy conservation with structural maintenance. This metabolic shift is consistent with previous findings in corals, where nutrient stress leads to reduced tissue expansion, reallocation of energy reserves, and altered metabolic activity (49).

Parallel to these morphological changes, starved polyps exhibited microbiome restructuring, characterized by decline in microbial diversity **(Figure 1-2A, B)** and a shift toward specific taxa **(Figure 1-3A)**. The loss of microbial richness suggests that only a subset of bacteria is capable of persisting under starvation, likely those metabolic flexibility or host-associated traits (21). In particular, Spirochaetota increased under starvation, while Gammaproteobacteria, including members of Vibrionaceae, declined **(Figure 1-3C, D)**. These results suggest that nutrient scarcity leads to the loss of transient, nutrient-dependent microbes while favoring a more stable and specialized microbial community, a pattern observed in other marine invertebrates undergoing prolonged environmental stress (11, 50, 51, 52). The stabilization of the microbiome under starvation may indicate a host-driven selection process, in which only bacterial taxa with beneficial interactions or resilience to resource depletion are maintained.

While starvation leads to microbiome stabilization through preferential survival of resilient bacterial taxa, frequent feeding supports microbial diversity without necessarily disrupting microbial homeostasis. Overfeeding studies in corals indicate that excessive nutrient input can disrupt microbial balance, promoting Gammaproteobacteria proliferation, increased bacterial loads, and higher pathogen susceptibility (13, 21)**.** However, in *Nematostella*, 5x/week feeding maintained microbial diversity **(Figure 1-2A)** while promoting continuous microbiome turnover **(Figure 1-2C, D)**, without triggering an overgrowth of resourceful taxa **(Figure 1-2B, 1-3A)**. Although frequent feeding sustains microbial diversity and promotes taxonomic shifts **(Figure 1-2A, 1-3A)**, starvation leads to a more streamlined

microbiome, likely reflecting an adaptation in which only beneficial or host-compatible taxa persist **(Figure 1-3C, D)**. These findings emphasize the ecological relevance of microbiome plasticity in *Nematostella*. Estuarine environments are characterized by periodic fluctuations in food availability, meaning that wild *Nematostella* must adapt to both starvation and nutrient excess like other marine invertebrates (53). The ability to dynamically regulate microbiome composition in response to feeding state may be an important strategy for maintaining holobiont resilience under variable environmental conditions.

Conclusion

This study offers new insights into how feeding and starvation affect both host morphology and microbiome composition in *Nematostella vectensis*. Starvation actively reshapes polyp morphology and microbial communities, causing fluctuations in polyp size, decreased microbial diversity, and selection for stress-tolerant bacterial taxa. Conversely, frequent feeding sustains microbial diversity and supports continuous microbiome dynamics without leading to microbiome collapse. Notably, polyps fed with germfree *Artemia* harbor significantly higher bacterial loads compared to conventionally fed polyps, highlighting an active, host-mediated process in microbial colonization.

Author contribution

NK, and SF conceptualized the study. NK and CW performed the experiments. NK performed 16S rRNA analysis and interpretation. NK wrote the first draft of the manuscript and SF contributed to manuscript revision.

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Chapter 2

cJUN dependent innate immunity controls microbiome through selective phagocytosis in *Nematostella*

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Abstract

Innate immunity, traditionally considered as a broad and non-specific defense system, is increasingly recognized for its capacity to selectively regulate microbial communities. Emerging evidence suggests that invertebrates can differentiate between closely related microorganisms, challenging the dichotomy between innate and adaptive immunity. This specificity is crucial for maintaining symbiotic relationships while preventing colonization by harmful microbes. In the sea anemone *Nematostella vectensis*, nematosomes—motile immune cells—play a key role in selective phagocytosis, preferentially engulfing foreign *Vibrio* isolates while sparing native bacterial colonizers. We identify the transcription factor cJUN as a crucial regulator of nematosome proliferation and function. CRISPR/Cas9-mediated knockout of *cJUN* resulted in a significant reduction of nematosome proliferation, impaired lysosomal activation, and an altered microbiome composition with increased colonization of non-native *Vibrio* strains. These findings demonstrate a direct link between host immune specificity and microbial community structure, challenging the traditional view of innate immunity as a non-specific defense. Our study highlights the evolutionary conservation of selective innate immune mechanisms and their role in maintaining microbial homeostasis in early-branching metazoans.

Introduction

A healthy functioning metaorganism, consisting of the host and its associated microorganisms, relies on an efficient immune system that recognizes, prevents and defends foreign threats, while controlling, regulating and maintaining homoeostasis. The immune responses of the host are based on a complex interplay of cells and mechanisms protecting the host from pathogenic microorganisms and foreign substances, like bacteria (1, 2), fungi (3) and viruses (4) while simultaneously regulating beneficial microbial relationships (5, 6). The widespread assumption is that the innate immunity provides an immediate and a non-specific defense, while the adaptive immunity enables a targeted, antigen-specific response with an immunological memory (7). Thereby, phagocytosis is a crucial innate immune defense mechanism in invertebrates and vertebrates. It involves the recognition and engulfment of foreign particles or altered self-cells (8, 9, 10) and consists of four main steps: target recognition, signaling for internalization, phagosome formation, and phagolysosome maturation (11, 12). The cellular mechanisms of invertebrate phagocytosis, including energy requirements and cytoskeletal involvement, are similar to those in mammalian macrophages (13). While professional macrophages are specialized for efficient phagocytosis in the innate immune system, enteric phagocytes in invertebrates also play a role in intracellular digestion of food particles (14). Although the fundamental mechanism of phagocytosis appears conserved between vertebrates and invertebrates, the evolutionary conservation of specific genes remains to be determined (9).

Recent research has challenged the traditional view that invertebrate immune systems lack specificity. Studies have shown that phagocytosis in invertebrates can exhibit a high degree of specificity (8, 15). In woodlice, hemocytes demonstrated increased phagocytosis of previously encountered bacterial strains, suggesting the ability to differentiate between closely related bacteria (8). In the squid-*Vibrio* symbiosis haemocytes are able to differentiate between the squid's preferred bacterial symbiont *Vibrio fisheri* and other bacteria of the *Vibrio* genus (16, 17). In addition, in a number of host-microbe interactions, it has been shown, that phagocytes can both shape the microbiota and be influenced by specific members of the microbiome. In tse-tse flies, for example, hemocyte proliferation depends on colonization by *Wigglesworthia* (18). A

similar effect is observed in pea aphids, where the presence of some symbionts affects hemocyte abundance and the proportion of granulocytes in the hemocyte population (19). The sea anemone *Nematostella vectensis (N. vectensis)*, a member of the phylum Cnidaria, provides a valuable platform to decipher the effects of innate immune specificity on interactions with its microbiome. *N. vectensis* exhibits a large genetic complexity, possessing most signaling pathways for development and immunity important in bilaterian animals (20, 21). *N. vectensis* completes its entire asexual and sexual life cycle under laboratory conditions. Thereby, its microbiome shows a specific succession during host development and robust adjustments to environmental variations while maintaining an ontogenetic core signature (22). In addition, the microbiome shows spatial structuring along the body column, and exhibits a diurnal pattern (23). Environmental influences, particularly temperature, play a significant role in shaping the microbiome composition, as well as host genotype, which contributes to bacterial community structure, with a notable genotype-environment interaction determining microbiota plasticity (24, 25). The microbiome-mediated plasticity (24), has been functionally linked to thermal adaptation in *N. vectensis* (25).

However, transcriptome data support the hypothesis, that the establishment of the microbiome strongly depends on selective mechanisms, like phagocytosis, that control the initial colonization processes (26). In *N. vectensis*, nematosomes (**Figure 2-1A**) are small motile multicellular bodies in the gastric cavity (27, 28), that originate from cnidoglandular tracts, the mesenteries, and consist of several different cell type (27).

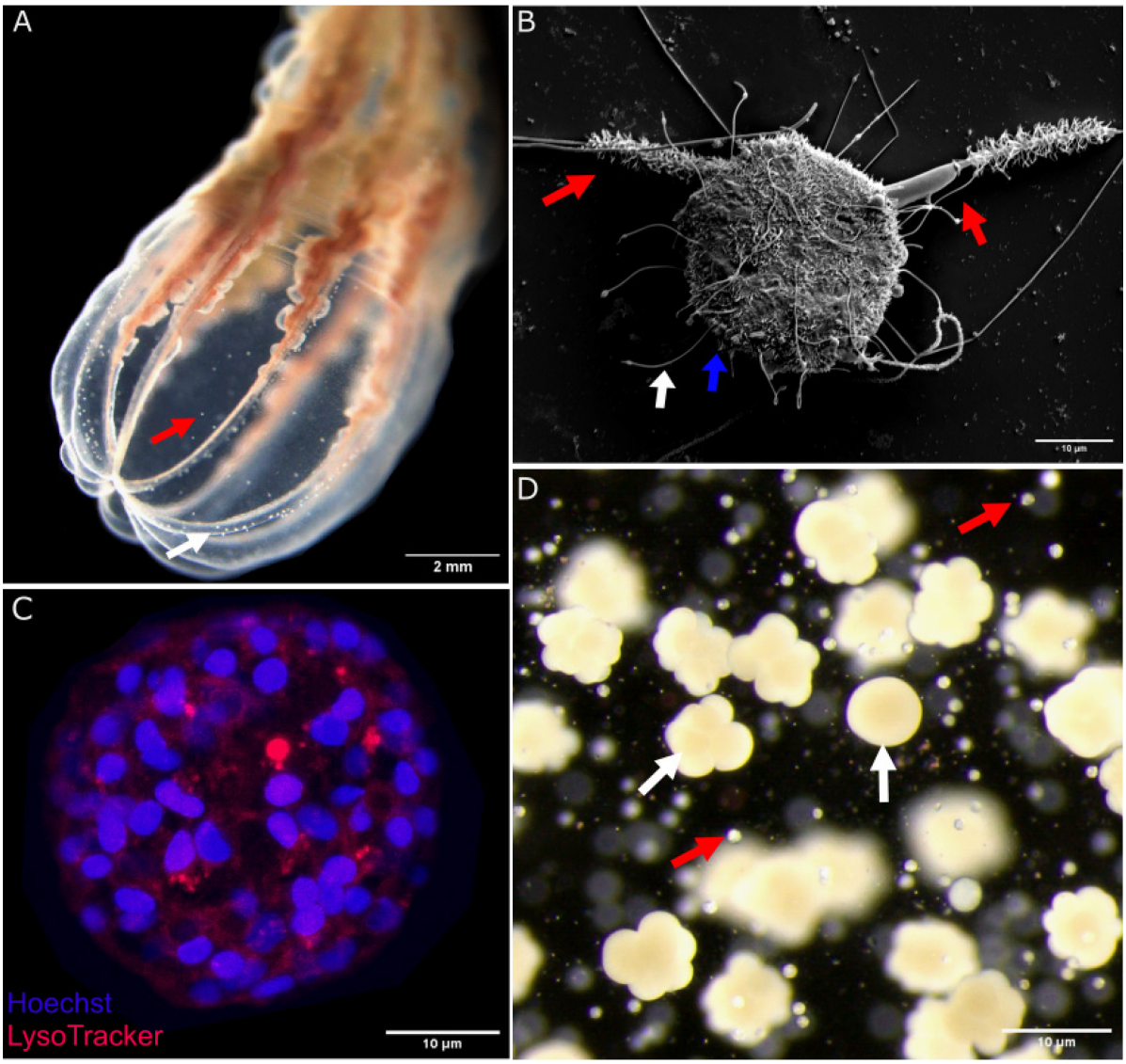

Figure 2-1 Nematosomes in and outside the polyp. (**A)** Foot region of adult *N. vectensis* polyp with nematosomes swimming inside the body cavity (red arrows) and nematosomes resting on the body wall of the polyp (white arrows). Scalebar represent 2 mm (**B)** SEM image of a single nematosome with two extending cnidocytes and discharged tubules (red arrows) and cilia highlighted with blue arrow (Type 1) and with a white arrow (Type 2) (27). (**C)** Confocal image of a nematosome stained with Hoechst (blue) and LysoTracker (red). (**D)** Egg package with eggs representing different cleavage status (white arrows) and nematosomes (red arrows) in between the eggs covered in the matrix. All scalebars represent 10 µm unless otherwise indicated.

They are equipped with two different types of cilia surrounding the cell complex (27), shorter type 1 cilia and longer type 2 cilia **(Figure 2-1B white and blue arrows)**, which facilitate mobility of the nematosomes. If nematosomes are not actively moving within the fluid of the gastric cavity, they locate along the inner body walls (**Figure 2-1A**).

Functionally, nematosomes can immobilize prey using cnidocytes (**Figure 2-1B**) and engulf bacteria through phagocytosis (**Figure 2-1C)**, suggesting a dual function in feeding and immunity (27, 28). A further indication for the active contribution to immunity of *N. vectensis* is the fact that nematosomes co-express components of the TLR signaling pathway, such as TLR and NF-kB (29). During oogenesis, nematosomes are packed into the egg clutches, which are expelled by the female polyps with a gelatinous matrix mucus that surrounds the eggs (**Figure 2-1D**).

Here, we propose the crucial role of cJUN in nematosome proliferation and function as microbial regulator in *N. vectensis*. Nematosomes exhibit selective phagocytosis, efficiently ingesting foreign *Vibrio* isolates and degrading them in the lysosome while sparing native *Vibrio* isolates. This selective phagocytosis is correlated with the ability of the bacteria to colonize *N. vectensis* adult polyps. Proteomic analyses revealed distinct protein enrichment patterns linked to phagosomal pathway in response to foreign bacteria, highlighting cJUN's role in immune-related trafficking. CRISPR/Cas9-mediated *cJUN* knockout (*cJUN*$^{-/-}$) resulted in a significantly reduction in nematosomes proliferation and impaired lysosomal activation after engulfment of *Vibrio* cells. These *cJUN*$^{-/-}$ polyps are colonized by an altered microbiome and accumulate foreign *Vibrio*-isolates, demonstrating a causal relationship between the composition of the microbiome and the selective phagocytosis of the nematosomes.

Methods

Nematostella vectensis culture

All experimental setups were conducted with adult clonal female *Nematostella vectensis* polyps, originally collected from the Rhode River in Maryland, United States (30, 31). The polyps were fed daily with freshly hatched *Artemia nauplii* and kept in dark. Culture boxes containing the animals, organized by genotype and gender, were connected to an aquatic system where the medium was flushed out and replaced with fresh Nematostella Medium (NM) with a salinity of 16‰ (Red Sea Salt® and Millipore H2O) at 18°C every other day. Every two weeks, the culture boxes were manually cleaned to remove biofilm and feeding debris.

Phagocytosis assay

Experimental setups regarding bacterial challenges were performed working with the same bacterial isolates used for mono-associations. Native (NJ1, NJ33 and NA11) and non-native (Hal025 and Hal281) bacterial isolates were grown at 30°C 220rpm overnight in liquid MB before diluting the isolates to an OD600 of 0.1/mL. 1mL of bacteria was centrifuged and diluted with sterile 16‰ NM prior staining with BacLight (Thermo Fisher) for 15 min in dark and room temperature. After incubation bacteria was centrifuged for one wash step with 16‰ NM before diluting them to OD600 of 0.001 for the bacterial challenge on the nematosomes ex vivo. Bacterial treatment was performed in the dark for 1.5 h at 18°C.

During bacterial staining, nematosomes from 5 clonal female polyps were extracted, by pinching a hole in the food region of the polyps and pipetting the discharged nematosomes, and were placed in a chamber slide (Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ System). After letting the cells stick to the bottom of the slide, they were washed once with NM to get rid of debris. The nematosomes were treated with stained bacteria from an OD600 of 0.001 and incubated for 2h in the dark. After bacterial treatment, nematosomes were washed 2 times with NM. After that nematosomes are stained with LysoTracker (15nM) and Hoechst (10nM) for 45 min at room temperature. Staining solution was washed out with NM after staining. Cells were than fixed with 3% PFA diluted in NM for 15min on RT and washed out once with NM after treatment.

Nematosomes were mounted on slide using ProLong™ Diamond (Thermo Fisher). Images from samples were taken with the confocal microscope Flouview 3000 and later analyzed using ImageJ (32).

Generation of germfree polyps and mono-association experiment

Antibiotic treatment (AB treatment) approaches were adapted from the established protocol for generating germfree *Hydra* polyps (33) and further adapted for *Nematostella* (34). Adult clonal *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ lines were exposed to a combination of five antibiotics: Ampicillin, Neomycin, Streptomycin, Spectinomycin, and Rifampicin, each at a concentration of 50 µg/mL. This treatment was conducted over a period of 2 weeks without any food supply, with the medium being refreshed every day and a replacement of plates every second day. For each treatment condition, five biological replicates were utilized, along with an additional five biological replicates serving as germfree (GF) and wildtype (WT) controls. Following the 2-week AB treatment, polyps were washed in sterile, filtered 16‰ NM before homogenization. A 1:10 dilution of the lysate was plated on Marine Broth (MB) agar plates to confirm sterility, in which GF plates should remain clear without bacterial growth. The remaining lysate was centrifuged, and the pellet was processed for DNA isolation using the Qiagen Blood and Tissue Kit for subsequent molecular analyses, including PCR and quantitative PCR (qPCR). After sterility confirmation remaining polyps were prepared for mono-association with chosen bacterial isolates. After 2 weeks of AB Treatment, polyps remained in sterile and filtered NM prior recolonization. Bacterial isolates were grown at 30°C in liquid MB media overnight. Bacteria was grown to an OD600 of 0,1, were diluted to a final OD600 of 0.001 and exposed to the sterile polyps. We used 5 polyps for each isolate and genotype of polyps with GF and WT control, respectively. The isolates we chose were NJ1, NJ33 and NA11 as native colonizers for *Nematostella* and Hal025 and Hal281 as non-native, foreign, colonizers, obtained from *Halichondria panicea*. After recolonizing polyps with single isolates, sampling took place after 2- and 7- days post recolonization (2dpr and 7dpr). Polyps were washed three times with 16‰ NM before getting homogenized and plated on MB plates. After an incubation time of 2 days on room temperature, colony forming units (CFU) were counted manually to determine the colonization succession of the single isolates on the polyp.

Proteomic analysis

Bacterial culture was prepared with an OD600 of 0.001 (see Phagocytosis assay) prior nematosomes extraction from adult polyps. All nematosomes from five biological replicates for each treatment was prepared. We chose the isolated NJ1 as a native colonizer and Hal281 as a non-native isolate and a control group without bacterial challenge. Extracted nematosomes were treated for 2h at 18°C with the isolates and were washed afterwards with 16% NM once. Nematosomes were transferred into a PCR tube and centrifuges by 5000rpm for 5min at 4°C to the bottom of the tube. Nm was discarded and replaced with 25µl Lysis Buffer (5mol/L Urea, 1% Tritonx100, 1xcOmpete EDTA-free, 5mmol/L DTT). Nematosomes were incubated for 45min at 37°C with vortexing in between every 15min. After incubation nematosomes were snapped-freezed at -80°C for further analysis.

Samples were then digested according to SP3 protocol (35) with some modifications as follows. After thawing, lysates were mixed each with 25 µL Alkylation buffer (50 mM borate buffer and 25 mM IAA) for 50 min at room temperature (RT). Then, 5 µL of resuspended SP3 beads (20 µg/µL A:B 1:1 mixture) were added to each sample followed by 150 µL ACN and mixed for 30 min at 800 rpm, RT. Then, beads were washed with 300 µL 70% EtOH, followed by 150 µL ACN. A 10 µL digestion buffer (4 ng/µL trypsin/Lys-C, 25 mM borate buffer and 0.01% DDOPM) was added to each sample followed by mixing on a shaker for 10 min at 800 rpm RT. Samples were mixed each by pipetting up and down and kept back on the shaker overnight. Next day, samples were centrifuged at 20,000 x g for 2 min and supernatant (ca. 10 µL) was transferred to LC-MS vials containing 1 µL of 5% FA.

LC-MS Proteomics and Data analysis

Chromatographic separation was performed on a Dionex U3000 nanoHPLC system equipped with an Acclaim pepmap100 C18 column (2 µm particle size, 75 µm × 500 mm) coupled online to a mass spectrometer. The eluents used were; eluent A: 0.05% formic acid (FA), eluent B: 80% ACN + 0.04% FA. The separation was performed over a programmed 120 minutes run. Initial chromatographic conditions were 4% B for 2 minutes followed by linear gradients from 4% to 50% A over 90 minutes then 50 to 90% A over 5 minute, and 10 minutes at 90% A. Following this, an inter-run equilibration of the column

was achieved by 16 minutes at 4% A. A constant flow rate of 300 nl/min was employed. Data acquisition following separation was performed on an QExactive Plus. Full scan MS acquisition was performed (350-1400 m/z, resolution 70,000). Subsequent data dependent MS/MS scans were collected for the 15 most intense ions (Top15) via HCD activation at NCE 27.5 (resolution 17,500); dynamic exclusion was enabled (20 sec duration). Triplicate measurements were performed for all the samples.

Raw data were analyzed against *Nematostella vectensis* Uniprot database (20.05.2022) (24,497 sequences) plus common contaminants (cRAP). The search was performed on Proteome discoverer 2.5 using a SequestHT search engine with 10 ppm and 0.02 Da precursor and fragment ions tolerances, respectively. Digestion with trypsin with a max of 2 missed cleavages were applied. Strict parsimony criteria have been applied filtering peptides and proteins at 1% FDR. INFERYs rescoring algorithm was applied. Label-free quantification method based on the intensities of the precursor ions was used. Proteins were filtered to have “High” FDR combined confidence and at least 2 identified peptides. Data was further analyzed by Excel and Perseus v 1.6.15.0 (36). Protein intensities were averaged for technical replicates to perform differential quantitative analysis of proteins, raw protein intensities were extracted, averaged between technical replicates, one outlier replicate per group “Control 5, Native 1 and Non-Native 2” were excluded, then median based normalization was applied to the data. Log2 transformed intensities were grouped in 3 groups depending on the Vibrio treatment (each with 5 replicates). Proteins with at least 4 intensity values in one group were used for further analysis. Missing values were imputed from a normal distribution separately for each replicate (Width 0.3, Downshift 1.8). Statistical analysis was done using ANOVA, permutation-based FDR of 0.01. Gene enrichment analysis was performed on The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (37, 38) using the functional annotation tool.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (ref).

CRISPR/Cas 9 mediated knock-out generation

CRISPR/Cas9-mediated transgenic lines were generated following the protocol published before (39). The gene *cJUN* (NVE21090) was selected based on transcriptomic analysis

after recolonization experiments (26) and tissue specificity (27). Four guide sequences (sgNVE21090E1, sgNVE21090E2, sgNVE21090E3, and sgNVE21090E4) were designed using the web tool CRISPOR.org (40), with an implemented Nematostella genome. First, the guide oligonucleotides were mixed in equal amounts, annealed for 5 min at 95°C, and then incubated at room temperature for 2-3 h. The annealed oligos were cloned into the gRNA expression vector pDR274 (42250, Addgene). After successful integration of the guides into the vector, the guide sequences were amplified, transcribed in vitro using the MEGAscript™ T7 kit (Thermo Fisher), and purified with the MEGAclear RNA cleanup kit (Thermo Fisher) prior to injection. The injection mix consisted of Cas9 enzyme (1 mg/ml stock) (TrueCut™ Cas9 Protein v2, Thermo Fisher), sgRNAs (450 ng), Alexa fluorescent dye (1.1 M in KCl), and RNase-free water. To obtain fertilized eggs, animals were incubated at 25°C for 11 h to induce gamete production. Egg packages were incubated in sperm media from males for 15 min before dejellying the fertilized egg packages with 4% cysteine (pH 7.4), followed by five washing steps in 16‰ NM. The injection mix was incubated at 37°C for 5 min before injection. The injection setup was conducted according to the microinjection protocol for mRNA and Morpholinos previously described (41). Injected eggs were raised in the dark at 20°C, with the medium (NM) being exchanged daily, and food introduction after 10-12 days.

To confirm the successful integration of CRISPR/Cas9-mediated mutagenesis into the polyps' genome, we performed crossbreeding, High Resolution Melting Curve Analysis (HRMC) and genotyping. Genomic DNA was isolated from injected juvenile polyps using the DNeasy Blood & Tissue kit (Qiagen). In HRMC, short DNA fragments at the targeted locus are amplified, and changes in these fragments are detected through shifts in the melting curves. Following confirmation of successful mutations, the mutant animals were crossed with wildtype polyps to generate the F1 generation. The same procedure was applied as for the previous F0 generation. Heterozygous animals were further analyzed by Sanger sequencing to determine the precise mutation pattern. Polyps with the same mutation pattern were crossed to generate the F2 generation, which included homozygous mutants (*cJUN*$^{-/-}$), homozygote wildtype (*cJUN*$^{+/+}$) and heterozygote (*cJUN*$^{+/-}$) offspring, which are used for experimental set ups.

Electron microscopy with nematosomes

Scanning electron microscopy (SEM) were taken with the Zeiss REM Supra 55VP. Nematosomes were extracted from the polyps and placed on well with Poly-L-Lysine coated cover glasses. All fixation, washing, dehydration were performed as former published (187). Images were taken with the Zeiss REM Supra 55VP and later analyzed using ImageJ.

16S rRNA analysis

For 16S rRNA analysis the bioinformatics were performed using Qiime 2 2021.11 (43). First, raw sequences were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with DADA2 (44). The amplicon sequence variants (ASVs) were aligned with mafft and for constructing the phylogeny fasttree2 was conducted (45, 46). All samples were rarefied to 900 sequences per samples prior estimation for Alpha-diversity metrics (observed features and Faith's Phylogenetic diversity (47)), beta-diversity metrics (Bray-Curtis dissimilarity, Jaccard distance and Unifrac (weighted and unweighted (48, 49))) and Principle Coordinate Analysis (PCoA). Taxonomy was assigned to ASVs using q2-feature-classifier classify-sklearn naïve Bayes taxonomy classifier against Greengenes 13_8 99% data set as reference (50). Further analysis, statistical analysis and plot visualization was conducted with OriginPro (Version 2021. OriginLab Corporation, Northampton, MA, USA.).

Results

Nematosomes phagocytose *Vibrio* strains with varying efficiency

To test if nematosomes are phagocytosing bacteria differentially we established a phagocytosis assay that allows to quantify on one hand lysosomal activity and on the other hand bacterial engulfment (**Figure 2-2A**).

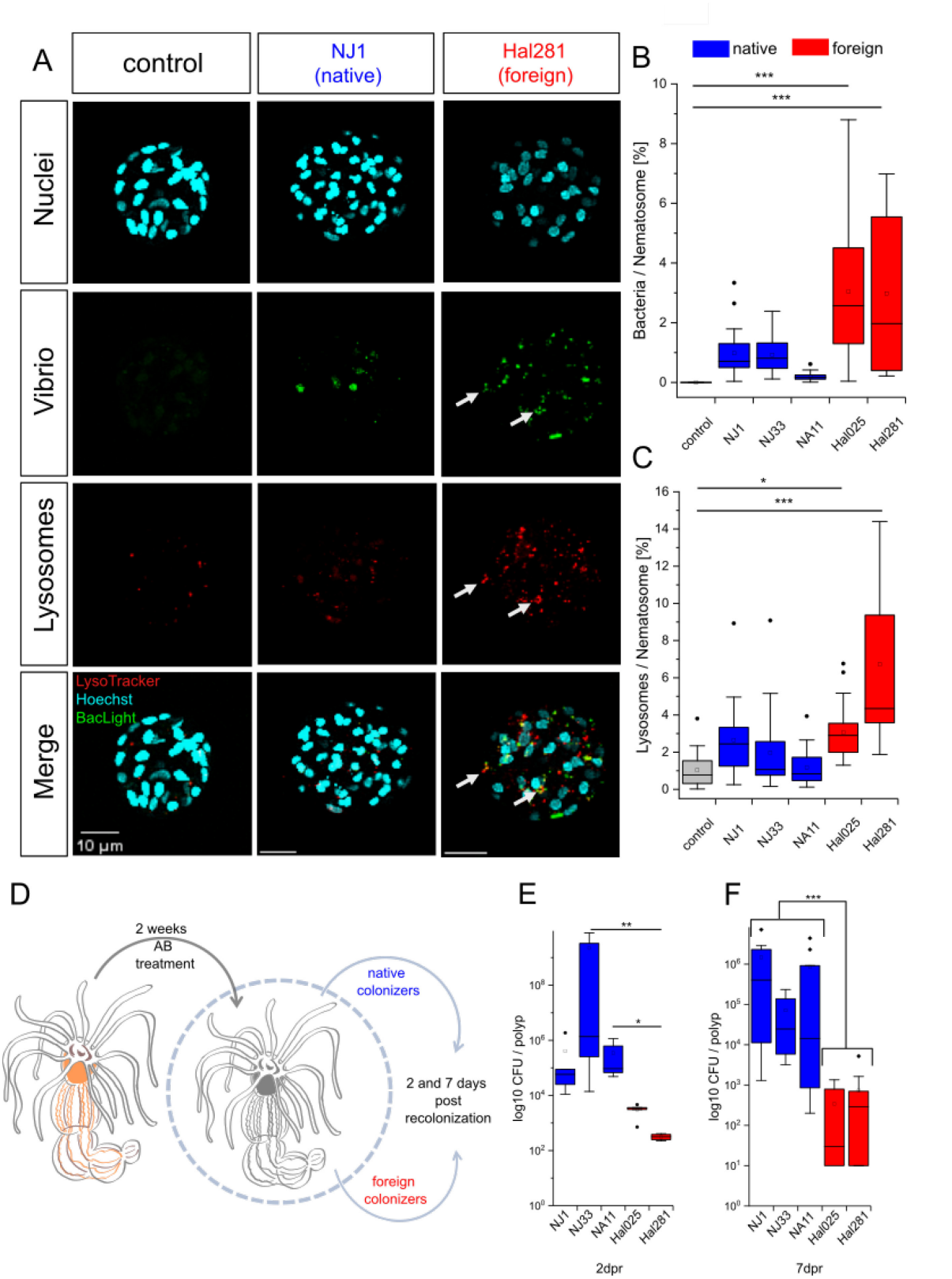

Figure 2-2 Nematosomes selectively phagocytose different *Vibrio* strains. (A) Confocal images of nematosomes challenged with NJ1 (native colonizer) and Hal281 (foreign colonizer). *Vibrios* are stained with BacLight and lysosomes with LysoTracker revealing that nematosomes are phagocytosing Hal281

more efficiently in comparison to native colonizer treatment and no bacterial challenge (control). Scalebars represent 10µm unless otherwise indicated. **(B)** Quantification of bacterial load per nematosome. The percentage of bacteria relative to the nematosome area shows significantly higher bacterial presence in nematosomes exposed to the foreign strains Hal025 and Hal281 compared to native strains and control. N= 10 - 22, Kruskal-Wallis ANOVA, * p<=0,05 ** p<=0,01 *** p<=0,001. **(C)** Lysosomal area relative to total nematosome area following bacterial challenge. Nematosomes exposed to the foreign strains exhibit a significantly larger lysosomal area compared to those treated with native strains and the control, indicating enhanced lysosomal activity. N= 10 - 22, Kruskal-Wallis ANOVA, * p<=0,05 ** p<=0,01 *** p<=0,001. **(D)** Schematic representation of the experimental setup. Adult *N. vectensis* polyps underwent a two-week antibiotic (AB) treatment to deplete resident microbiota, followed by mono-association with either native or foreign *Vibrio* strains. Colonization was assessed after seven days post-recolonization (dpr). **(E)** Quantification of *Vibrio* colonization levels in adult polyps 2 dpr. Colony-forming units (CFU) per polyp are significantly lower for foreign strains compared to native colonizers, indicating reduced colonization efficiency of the foreign strains. N=5 polyps each, One-way ANOVA revealed significant differences between the groups native (blue) and foreign (red) isolates, * p<=0,05 ** p<=0,01 *** p<=0,001. **(F)** Quantification of *Vibrio* colonization levels in adult polyps 7 dpr. Colony-forming units (CFU) per polyp are significantly lower for foreign strains compared to native colonizers, indicating reduced colonization efficiency of the foreign strains. N=5 polyps each, Two-way ANOVA revealed significant differences between the groups native (blue) and foreign (red) isolates, * p<=0,05 ** p<=0,01 *** p<=0,001.

Therefore, nematosomes are extracted from the gastric cavity and incubated in a bacterial suspension with defined bacterial concentration. For the assay we chose different *Vibrio* strains, as they are common marine bacteria and are among the main colonizers of *N. vectensis.* As native isolates we selected the *Vibrio* isolates NJ1, NJ33 and NA11 that were cultivated from *N. vectensis* **(Table 2-1)** (26). We analyzed the phagocytosis rate of these native isolates and compared it with the rate of phagocytosis of foreign *Vibrio* isolates (Hal025 and Hal281) derived from the sponge *Halichondria panicea* **(Table 2-1)**.

Table 2-1 Identification and closest type strain matches of *Vibrio* isolates from *Nematostella vectensis* and *Halichondria panicea*. 16S rRNA gene sequences from five bacterial isolates were compared to type strain sequences using BLAST. Each isolate's host species, sequence length, GenBank accession number (if available), and closest type strain matches with corresponding sequence IDs and percent similarity are listed.

Isolate	Host	Sequence length (GenBank accession number)	Closest type strains (with Sequence ID)	Similarity (%)
NJ1	*Nematostella vectensis*	1455 bp (PQ455196)	*Vibrio diazotrophicus* (MT406422.1) *Vibrio plantisponsor* (AP024893.1)	99% 99%
NJ33	*Nematostella vectensis*	775 bp	*Vibrio anguillarum* (NR_042509.1) *Vibrio ziniensis* (NR_181540.1)	95% 95%
NA11	*Nematostella vectensis*	1008 bp	*Vibrio vulnificus* (NR_036888.1) *Vibrio fluvialis* (NR_036790.1)	97% 96%
Hal025	*Halichondria panicea*	1545 bp	*Vibrio artabrorum* (NR_116068.1) *Vibrio celticus* (NR_116066.1)	99% 99%
Hal281	*Halichondria panicea*	1517 bp (MT406665)	*Vibrio atlanticus* (NR_116067.1) *Vibrio cyclitrophicus* (NR_115806.1)	99% 99%

The results revealed that native *Vibrio* strains were phagocytosed at significantly lower rates, while the foreign isolates Hal025 and Hal281 were engulfed at substantially higher rates **(Figure 2-2A, B)**. The increased phagocytosis of foreign isolates correlated with significant increase in lysosomal activity within nematosomes **(Figure 2-2A, C)**. In contrast, nematosomes confronted with native *Vibrio* strains did not increase their lysosomal activity (**Figure 2-2A, C**).

In addition to the phagocytosis rate for each isolate, we also compared the colonization efficiency of each isolate in mono-association experiments (**Figure 2-2D, E, F**). This recolonization approach revealed that the three native *Vibrios*, namely NJ1, NJ33 and NA11, colonized in significantly higher rates on the polyp 7 days post recolonization (dpr), compared to the two foreign isolates Hal025 and Hal281 (**Figure 2-2E**). This trend was already seen after 2 dpr (**Figure 2-2F**). These results correlate with the observations of elevated phagocytosis rates (**Figure 2-2B**) and lysosomal actives (**Figure 2-2C**) of the nematosomes upon foreign *Vibrio* engulfment suggesting a potential link between nematosome phagocytosis and colonization success.

Foreign and native *Vibrio* isolates cause diverging proteome responses in nematosomes

To characterize the differential response of nematosomes to native and foreign *Vibrio* strains, we performed proteome analysis with extracted nematosomes. Specifically, we assessed the responses of nematosomes after confronting them with NJ1 and Hal281, and compared it to a control treatment. The proteome analysis revealed significantly abundant proteins when comparing nematosomes challenged with native and foreign bacterial isolates (**Figure 2-3, 2-4**).

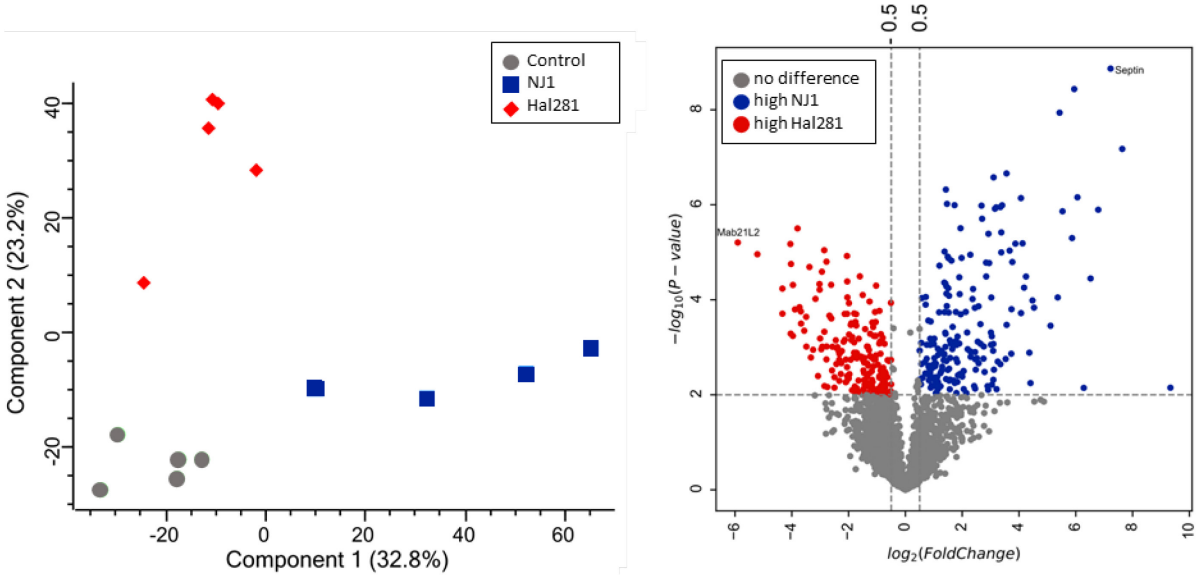

Figure 2-3 Proteomic response of nematosomes following treatment with bacterial isolates NJ1 and Hal281. **(A)** Principal component analysis (PCA) of proteomic profiles showing distinct clustering patterns among nematosomes treated with isolates NJ1 (blue), Hal281 (red), and control nematosomes without bacterial challenge (grey). Percentages indicate the explained variance for each principal component. **(B)** Volcano plot illustrating differentially abundant proteins identified between NJ1- and Hal281-treated nematosomes. Proteins significantly enriched ($p<0.05$, $\log_2$ fold-change > 0.5) in NJ1-treated nematosomes are shown in blue, those enriched in Hal281-treated nematosomes are in red, and proteins with no significant difference are represented in grey. Selected proteins with strong differential expression are labeled explicitly on the plot.

A total of 2676 proteins were detected in the proteomic analysis of nematosomes treated with bacterial isolates NJ1 and Hal281, as well as in untreated controls. To extract proteins that were uniquely differently abundant in either NJ1 or Hal281 treatment, we generated five clusters using *k*-means clustering (**Figure 2-4A**). Out of the total proteins identified, 157 proteins were detected uniquely in NJ1-treated samples and 104 in Hal281-treated

nematosomes **(Figure 2-4B)**. Thereby, cluster 1 represents proteins which emerged exclusively following NJ1 treatment, while cluster 4 contain proteins, which are higher in abundance after confrontation with Hal281. A KEGG enrichment analysis revealed that cluster 1, which contains proteins that are significantly more abundant in NJ1-treated nematosomes, contains proteins related to carbon and nitrogen metabolism **(Table 2-S1)**. These findings suggest that interaction with native bacteria may promote the host metabolisms, reflecting potential symbiotic interactions. In contrast, that treatment with Hal281 elevated the abundance of proteins belonging to the phagosomal pathway (**Table 2-S1**). Especially proteins belonging to the cytoskeleton formation of phagosomal formation, like Dynein and Tubulin beta (TUBB) as well as F-actin are increased (**Figure 2-4C**). Interestingly, the V-ATPase shows an increase in abundance in both bacterial treatments, potentially linking it to default lysosomal activity (**Figure 2-4C**).

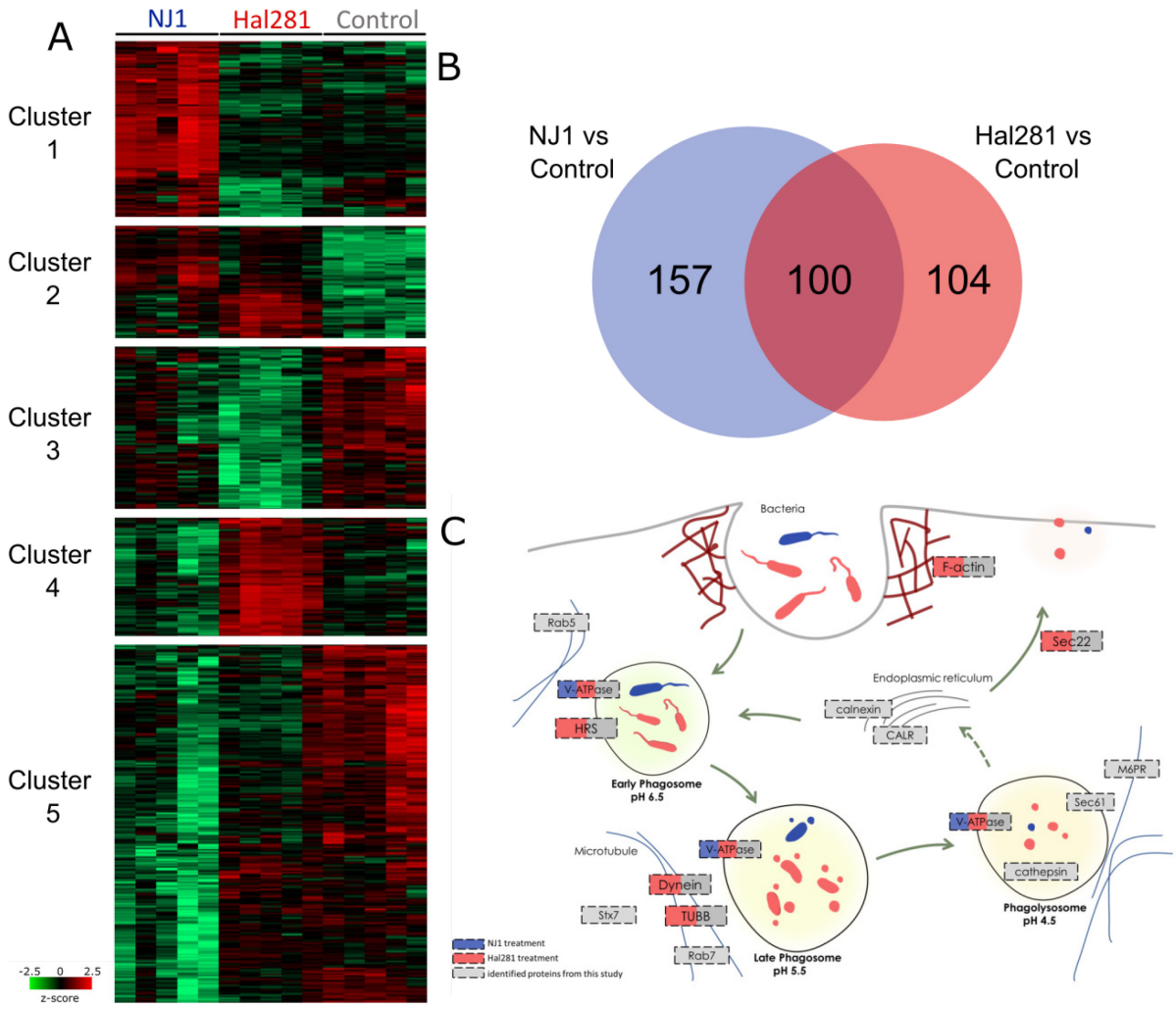

Figure 2-4 Differentially abundant proteins in native vs foreign treatment in nematosomes. This study was conducted with five biological replicates per group: a control group, a group treated with a native isolate (NJ1) and a group treated with a foreign isolate (Hal281). A total amount of 2676 proteins were detected, 257 proteins highly abundant in the native isolate-treated group, 204 proteins highly abundant in the foreign isolate-treated group, and 77 proteins highly abundant in the untreated control group. **(A)** Heatmap showing clustering of differentially abundant proteins across three conditions: nematosomes treated with NJ1, Hal281, and untreated control. Five distinct protein clusters are identified base on functional enrichment. Cluster 1: oxidative phosphorylation and carbon metabolism, Cluster 2 amino acid metabolism, Cluster 3 amino acid degradation and metabolic pathways, Cluster 4 Phagosome, and Cluster 5 Ribosome. Protein abundance is represented as Z-scores, with red indicating higher and green lower abundance. **(B)** Venn diagram illustrating the proteins identified uniquely in NJ-treated (157 proteins) and uniquely in Hal281-treated (104 proteins), and 100 proteins shared between NJ1 and Hal281 treatments. **(C)** Schematic representation of the phagosome-lysosome pathway in nematosomes following bacterial exposure. The diagram shows the proposed trafficking routes from bacterial uptake, early phagosome formation, lysosomal fusion, and subsequent degradation (47, 48, 49). Proteins significantly enriched in NJ1-treated (blue) and

Hal281-treated (red) nematosomes are mapped onto corresponding pathway components, suggesting differential regulation of lysosomal processing depending on the bacterial isolate.

The proteomic analysis revealed an elevated abundance in the phagosomal pathway upon foreign bacterial treatment, indicating an active response of nematosomes in immune response, particularly in recognizing foreign isolate phagocytosis and degradation. To further explore the molecular mechanisms driving these responses, we aimed to manipulate the regulatory pathways involved.

cJUN mutation reduces nematosome proliferation

In a next step we aimed to alter the function of nematosomes by CRISPR/Cas9 genome editing. Therefore, we screened for potential transcription factors (TF) that are potentially involved in the proliferation and/or immune function of nematosomes. We identified *cJUN* orthologs, which is highly expressed in nematosomes (NVE21090) (**Figure 2-5A**), while a second ortholog is mainly expressed in the tentacle region (NVE16876) (**Figure 2-5B**) of the polyp (26, 27). Interestingly, the *cJUN* ortholog NVE21090 is also upregulated upon bacterial recolonization (26). *cJUN* is an evolutionary conserved transcription factor with a central role in activation of inflammatory pathways, regulating phagocytotic activity and is involved in cell proliferation (54, 55, 56). As central hub, cJUN integrates signaling information of various pathways, including ERK and JNK signaling (57). In the cnidarian Hydra TLR signaling via MyD88 activates JNK signaling following immune stimulation (58) and in vertebrates cJUN regulates macrophage activation (59, 60).

Therefore, we selected the *cJUN* ortholog NVE21090 for CRISPR/Cas9 genome editing (39), to be able to functionally investigate the role of nematosomes in regulating microbiome composition. We generated deletions in the first exon of NVE21090, which ultimately led to a variation of mutations in animals of the F1 generations (**Figure 2-4C**). For the subsequent breeding, we selected the male strain M8 and the female strain M11, both carrying the same heterozygous mutation leading to a stop codon in the first exon of the gene. This mutation results in incomplete translation of the gene, leading to the loss of essential domains within the protein (**Figure 2-4E)**. All subsequent approaches were conducted on F2 polyps with homozygous mutation named *cJUN*$^{-/-}$ (**Figure 2-4D**).

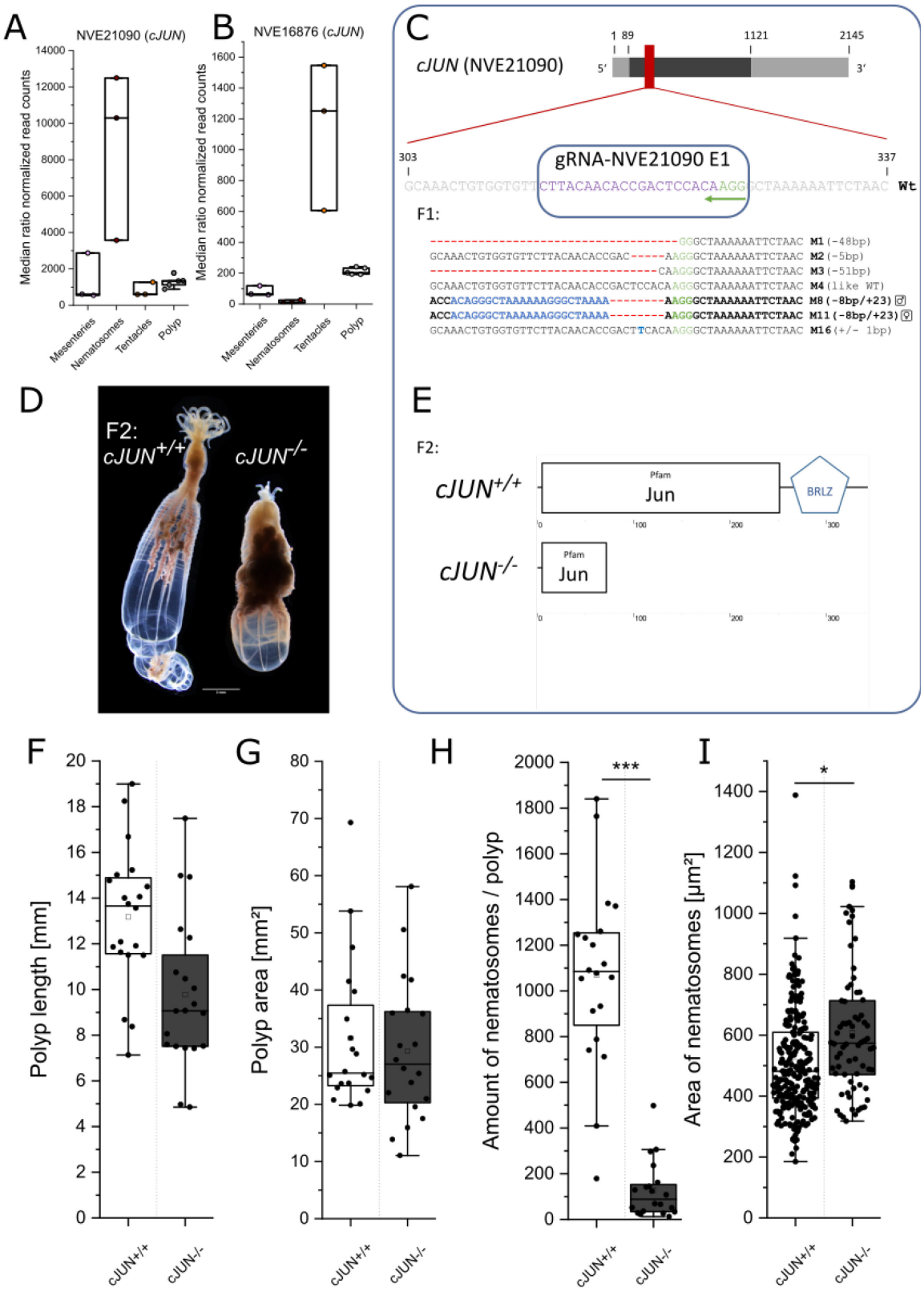

Figure 2-5 NVE21090 mutations leads to stop codon in the upstream region of exon. (A) NVE21090 *(cJUN)* expression on mesenteries, nematosomes, tentacles and whole polyp (26, 27). **(B)** NVE16876 *(cJUN)* expression across the same tissues. Median ratio normalized read counts are represented, showing

tissue specific expression patterns (26, 27). **(C)** Schematic illustration of NVE21090 (*cJUN*) locus with guide recognition sites (UTR in bright grey and exon in dark grey). The F1 generation displays a range of mutations, including deletions (red), insertions (blue), and the PAM sequence (green). The guide sequence is highlighted in purple. F2 with highlighted homozygous mutation leading to a stop codon (bold). Additional predicted SMART domains missing in F2 mutant animals upon confirmed mutation pattern. **(D)** F2 adult polyps. Left *cJUN*$^{+/+}$ and right *cJUN*$^{-/-}$. **(E)** Scheme of protein structure of wildtype and cJUN mutant F2 animals with essential domains missing in *cJUN*$^{-/-}$ (generated with SMART). **(F)** No significant differences were observed in polyp length between *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals. **(G)** Polyp area also remained comparable between genotypes. **(H)** The number of nematosomes per polyp was significantly reduced in mutant animals. N=50-250, two-sample t-test, * p<=0,05 ** p<=0,01 *** p<=0,001. **(I)** The area of nematosomes in *cJUN*$^{-/-}$ polyps was slightly larger compared to wildtype nematosomes. N=50-250, two-sample t-test, * p<=0,05 ** p<=0,01 *** p<=0,001.

Morphological comparisons between *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ polyps revealed no significant differences in polyp length (**Figure 2-5D, F)**. Simultaneously, *cJUN*$^{-/-}$ polyps showed no significant differences in area size, indicating that *cJUN*$^{-/-}$ mutation in nematosomes leads to no significant changes in polyps' body (**Figure 2-5D, G**). However, a significant reduction in nematosome numbers per polyps was observed (**Figure 2-5H**). While adult *cJUN*$^{+/+}$ polyps harbor around 1100 nematosomes, *cJUN*$^{-/-}$ polyps only exhibit around 100 per polyp. Interestingly, this lower number seems to be slightly compensated by size, as *cJUN*$^{-/-}$ nematosomes display a bigger size compared to *cJUN*$^{+/+}$ nematosomes (**Figure 2-5I**).

cJUN mutation affects nematosome phagocytosis and bacterial colonization

To approach the effect of cJUN on nematosome phagocytosis, we performed the newly established phagocytosis assay, confronting *cJUN*$^{-/-}$ and *cJUN*$^{+/+}$ polyps with the same bacterial isolates (**Figure 2-6A**). *cJUN*$^{+/+}$ revealed similar lysosomal activity and rates of phagocytosis as observed in wt animals after confrontation with foreign and native *Vibrio* strains (**Figure 2-2B, C and Figure 2-6A, B, C)**. However, while *cJUN*$^{+/+}$ nematosomes adjust their lysosomal activity in response to different isolates, *cJUN*$^{-/-}$ nematosomes maintain a similar default lysosomal activation independent of bacterial treatment (**Figure 2-6C**). Being confronted with the foreign isolates Hal025 and Hal281 was not resulting in the activation of lysosomal activity in the *cJUN*$^{-/-}$ nematosomes. Analyzing the number of

bacteria engulfed in nematosomes revealed an increase of the isolates NA11 and Hal281 (**Figure 2-6B**) in *cJUN*$^{-/-}$ nematosomes, most likely by an accumulation of bacterial cells in the phagosome. As the lysosome is not activated in *cJUN*$^{-/-}$ nematosomes, degradation of bacteria in phagosome is most likely impaired, resulting in arrested phagocytosis.

The impaired phagocytosis of foreign *Vibrio* isolates resulted in an increased recolonization rate in mono-association experiments (**Figure 2-6D, E**) in adult *cJUN*$^{-/-}$ polyps compared to *cJUN*$^{+/+}$ polyps (**Figure 2-6E**). Interestingly, the native isolates NJ1 and NJ33 recolonized *cJUN*$^{-/-}$ polyps significantly lower compared to *cJUN*$^{+/+}$ polyps, suggesting even supporting effects of nematosomes for some native colonizers.

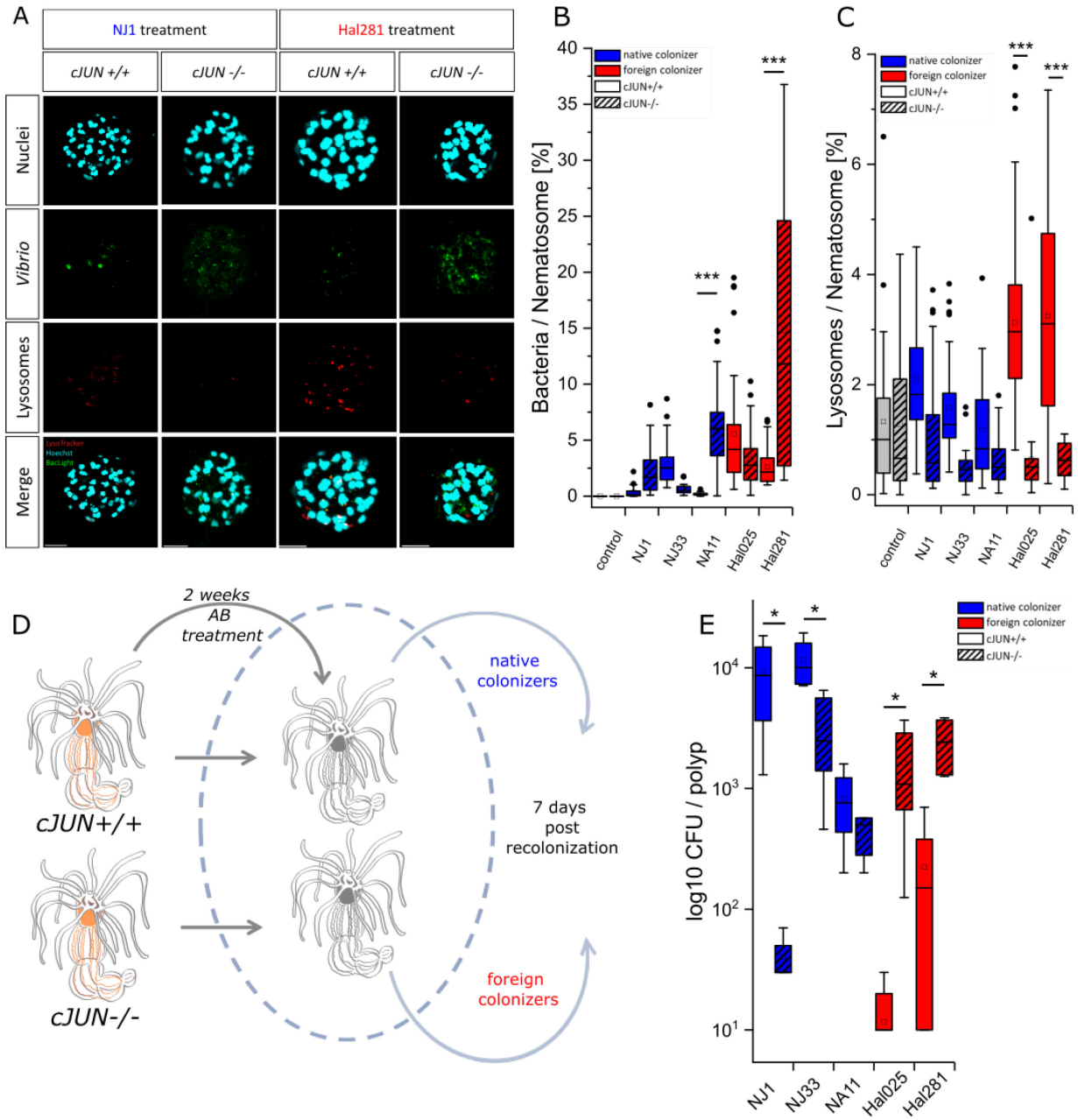

Figure 2-6 *cJUN* depleted nematosomes are not able to recognize *Vibrio* strains. (A) Confocal images of *cJUN* depleted and not depleted nematosomes challenged with NJ1 and Hal281. *Vibrio* staining with BacLight and lysosome staining with LysoTracker reveal that nematosomes are phagocytosing Hal281 more efficiently in comparison to no bacterial challenge and native colonizer treatment. Scalebars represent 10µm unless otherwise indicated. **(B)** Quantification of bacterial engulfment per nematosome. The percentage of bacteria relative to nematosome area reveals significantly higher bacterial presence in *cJUN*$^{-/-}$ upon Hal281 treatment compared to *cJUN*$^{+/+}$ nematosomes and native isolate treatment. N= 30-70, Kruskal-Wallis ANOVA, * p<=0,05 ** p<=0,01 *** p<=0,001. **(C)** Relative lysosomal activity per nematosome following bacterial challenge. Lysosomal area is significantly higher in *cJUN*$^{+/+}$ nematosomes exposed to Hal281 compared to *cJUN*$^{-/-}$ nematosomes and native isolate treatments. N= 30-70, Kruskal-Wallis ANOVA, *

p<=0,05 ** p<=0,01 *** p<=0,001. **(D)** Simplified illustration of AB treatment before mono-association with native and foreign *Vibrio* strains. **(E)** Mono-association of native and foreign colonizers on adult polyps after 7 days post recolonization. N=5 polyps each, Kruskal-Wallis ANOVA, * p<=0,05 ** p<=0,01 *** p<=0,001.

Bacterial dysbiosis in *cJUN*$^{-/-}$ polyps

Discovering significant differences in the phenotype of *cJUN*$^{-/-}$ polyps, we proceeded to analyze its associated microbiome. 16S rRNA gene sequencing revealed significant differences between the microbiome of *cJUN*$^{-/-}$ and *cJUN*$^{+/+}$ polyps (**Figure 2-7, Table 2-2**), with a significantly lower alpha diversity (**Figure 2-7C**) and evenness (**Figure 2-7D**) compared to *cJUN*$^{+/+}$ animals.

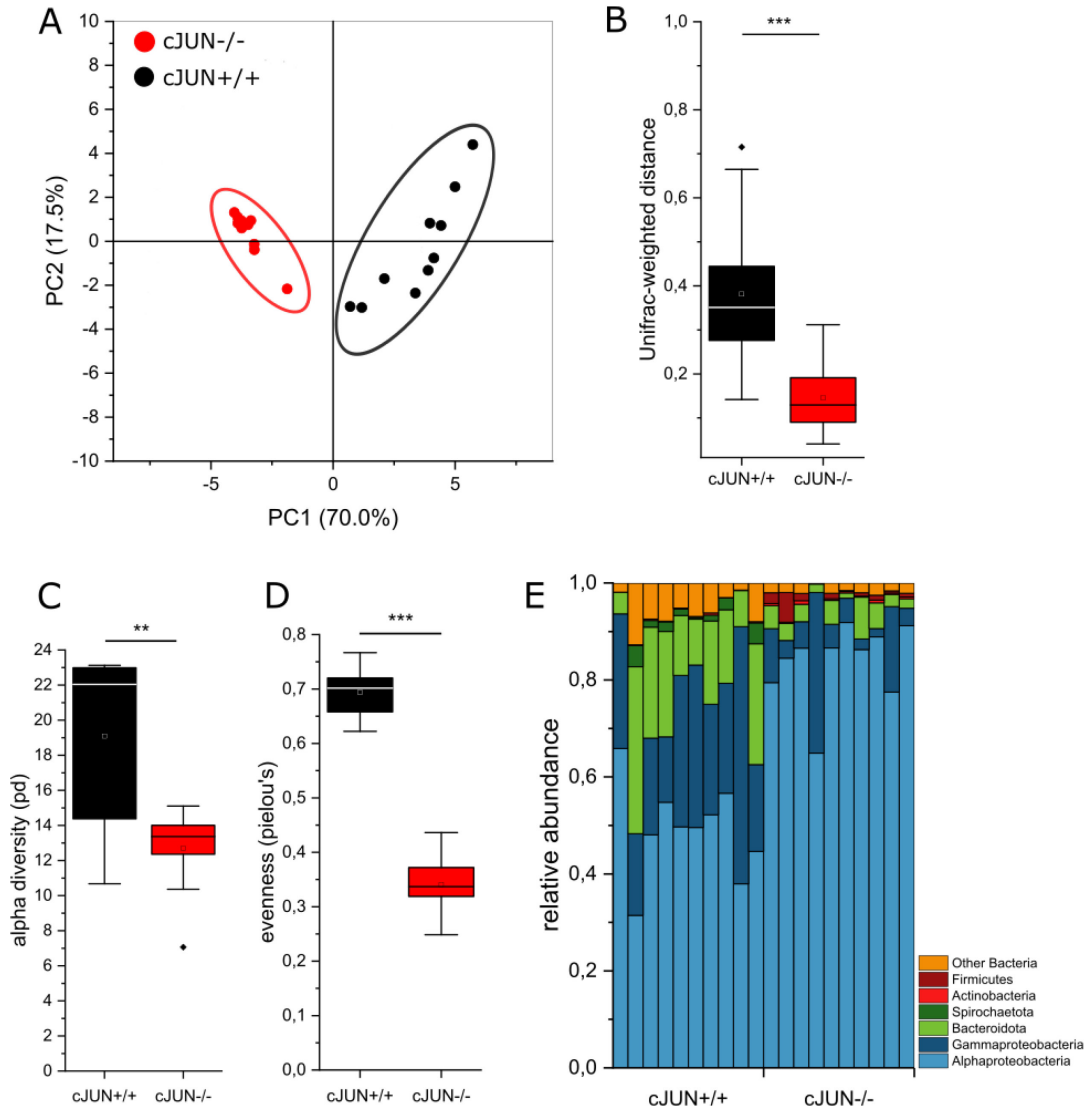

Figure 2-7 Impact of *cJUN* KO on microbial diversity and abundance of specific taxa in *Nematostella* polyps. (A) Principal Coordinate Analysis (PCoA) plot based on weighted UniFrac distances, comparing the microbial communities of *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ polyps. The two groups form distinct clusters, indicating

significant differences in microbial community composition. **(B)** Weighted UniFrac distance demonstrates distinct microbial community composition in *cJUN*$^{-/-}$ versus *cJUN*$^{+/+}$ polyps. **(C)** Alpha diversity analysis using Faith's Phylogenetic Diversity (PD) index revealed a significant reduction in microbiome richness in *cJUN*$^{-/-}$ polyps compared to *cJUN*$^{+/+}$ polyps. N=20/genotype, two-sample t-test, * p<=0,05 ** p<=0,01 *** p<=0,001. **(D)** Microbial community evenness (Pielou's evenness index) significantly decreases in *cJUN* mutant polyps compared to wildtype control. **(E)** Bar plots representing the relative abundance of bacterial taxa in *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals. Each bar shows the mean relative abundance of taxa, including Alphaproteobacteria, Gammaproteobacteria, Bacteroidota, Spirochaetota, Actinobacteria, and Firmicutes, across samples for each genotype, highlighting differences in the microbial community composition between the two groups.

Table 2-2 Statistical summary of ADONIS and ANOSIM tests on Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac distance matrices, comparing microbial community dissimilarities between *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals. The analysis was performed at the genotype level. Adonis R^2 values represent the proportion of variance explained by genotype while ANOSIM R values indicate the degree of separation between groups. Significant differences are indicated by p-values, with higher R values reflecting stronger microbial dissimilarities between groups.

Parameter	Metric	Adonis R^2	Adonis p	ANOSIM R	ANOSIM p
genotype	Bray-Curtis	0.51	<0,001	0.81	<0,001
	Jaccard	0.36	<0,001	0.90	<0,001
	Weighted UniFrac	0.59	<0,001	0.84	<0,001
	Unweighted UniFrac	0.43	<0,001	0.82	<0,001

These differences in bacterial colonization in *cJUN*$^{-/-}$ and to *cJUN*$^{+/+}$ polyps support previous results indicating that host mechanisms are involved in the control of bacterial establishment in *N. vectensis* (24). To test this hypothesis, we recolonized germfree adult *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ polyps with the bacterial consortia of adult polyps and followed the succession of bacterial establishment over the period of 28 days by 16S rRNA gene sequencing. Over the whole course of the experiment the different genotypes have significant effects on microbial community structure (**Table 2-3, Figure 2-8D**). In contrast to cJUN, dpr accounted for a greater proportion of microbial variability than genotype **(Table 2-3)**.

Table 2-3: Statistical summary of ADONIS and ANOSIM tests on Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac distance matrices, comparing microbial community dissimilarities between *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals. The parameter column indicates whether the analysis was performed at the genotype level or at the dpr level. Adonis R^2 values represent the proportion of variance explained by genotype or dpr while ANOSIM R values indicate the degree of separation between groups. Significant differences are indicated by p-values, with higher R values reflecting stronger microbial dissimilarities between groups.

Parameter	Metric	Adonis R^2	Adonis p	ANOSIM R	ANOSIM p
genotype	Bray-Curtis	0.097	0.005	0.17	0.002
	Jaccard	0.099	<0.001	0.31	<0.001
	Weighted UniFrac	0.073	0.031	0.14	0.007
	Unweighted UniFrac	0.101	<0.001	0.23	<0.001
dpr	Bray-Curtis	0.174	<0.001	0.42	<0.001
	Jaccard	0.111	<0.001	0.40	<0.001
	Weighted UniFrac	0.282	<0.001	0.49	<0.001
	Unweighted UniFrac	0.110	<0.001	0.28	<0.001

During the early timepoints (2 and 7 dpr) *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ polyps exhibit similar patterns in microbial richness and evenness (**Figure 2-8A, B**). After 14 dpr, the microbiome of *cJUN*$^{+/+}$ polyps show significantly higher bacterial alpha diversity and evenness compared to *cJUN*$^{-/-}$ polyps **(Figure 2-8A, B)**. The increase in Weighted UniFrac distances between the microbiome of the different genotypes reveal that genotype-related differences in the composition of the microbiome become more pronounced over time, as the microbial communities develop differently between *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals (**Figure 2-8C**).

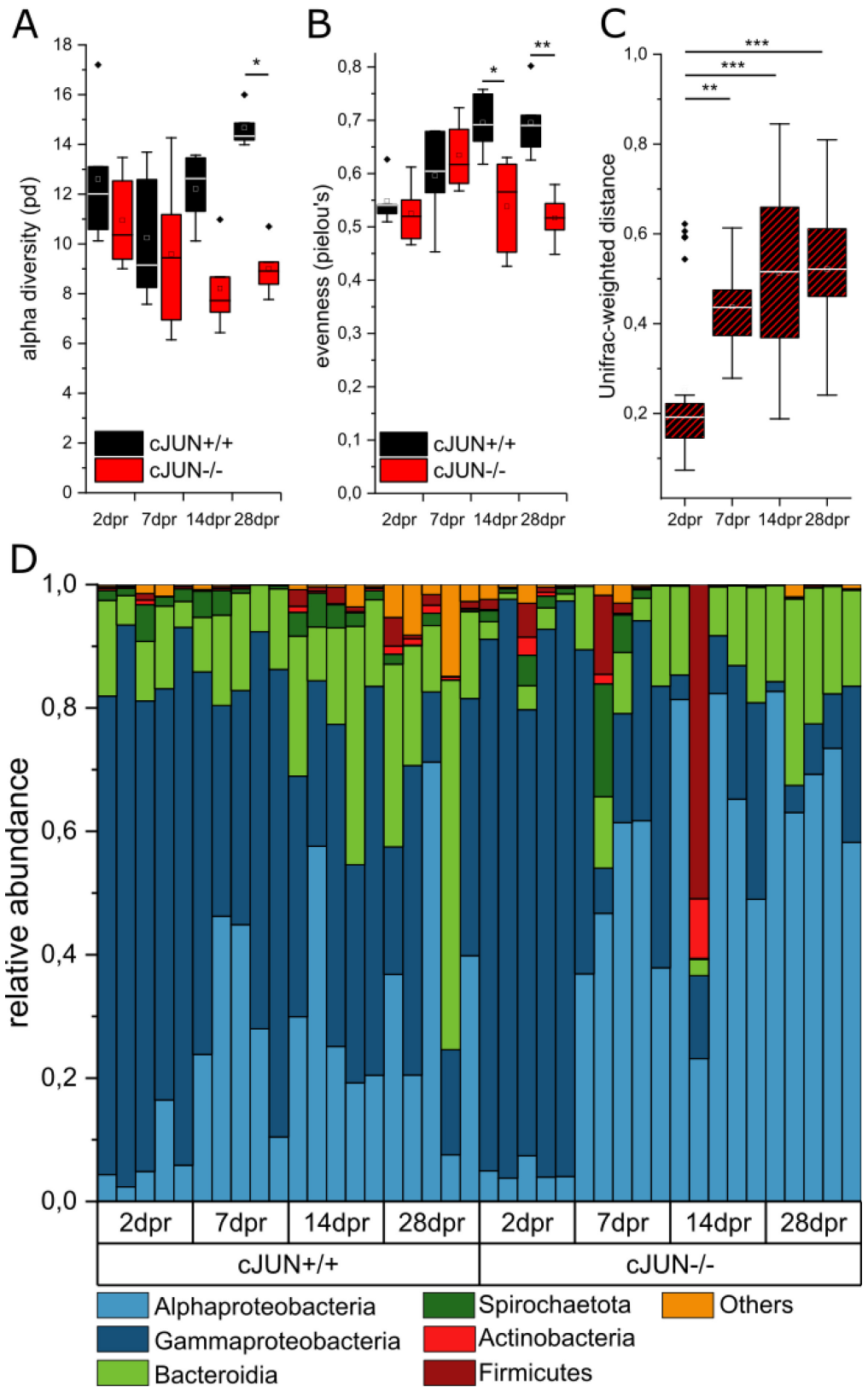

Figure 2-8 Microbiome comparison between *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ after recolonization. **(A, B)** Alpha diversity metrices for *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ microbiome comparison over a time course of 1 month (2,7,14,28 dpr). Both genotypes display similar values for richness (A) and evenness (B) after 2 and 7 dpr and separate

after 14 dpr. *cJUN*$^{+/+}$ animals show a stable and rich microbiome composition while *cJUN*$^{-/-}$ reveal low level in Faith's PD and evenness. N=5, Kruskal-Wallis ANOVA, * p<=0,05 ** p<=0,01 *** p<=0,001. **(C)** Weighted UniFrac distance comparisons between microbial communities of *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ polyps over time, Kruskal-Wallis ANOVA, * p<=0,05 ** p<=0,01 *** p<=0,001. **(D)** Bar plots representing the relative abundance of bacterial taxa in *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals over time. Each bar shows the mean relative abundance of taxa across samples for each genotype, highlighting differences in the microbial community composition among the two groups and in the timepoints after recolonization.

The significant differences in specific microbial taxa observed between the genotypes over time (**Figure 2-9**), including the dominance of some Alphaproteobacteria taxa (**Figure 2-9A, B**) and the reduced colonization success of specific Gammaproteobacteria (**Figure 2-9D**) highlight the selective force of nematosomes on specific taxa.

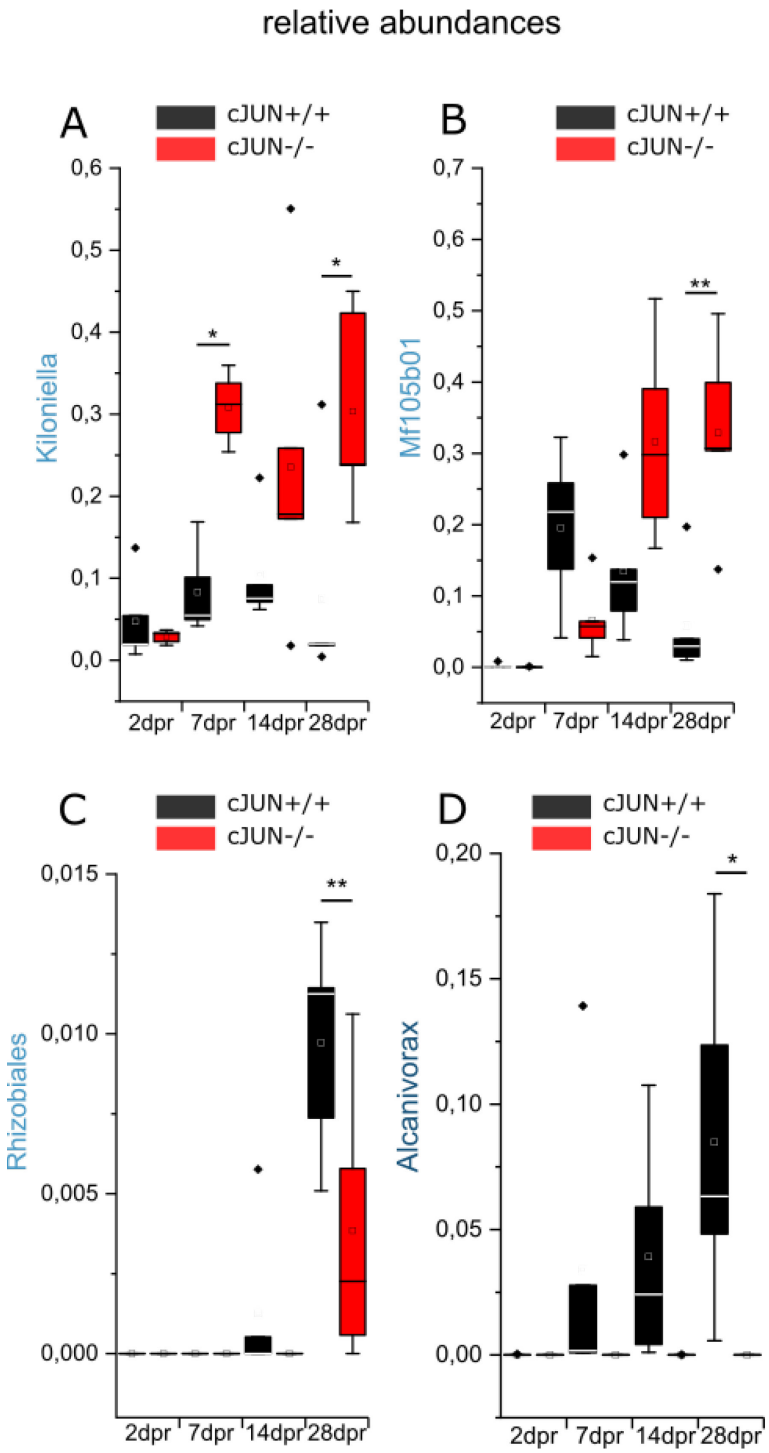

Figure 2-10 Temporal dynamics of differentially abundant bacterial taxa in *cJUN*$^{-/-}$ and *cJUN*$^{+/+}$ polyps. Relative abundances of specific bacterial taxa over time (2, 7, 14, and 28 days post-recolonization [dpr]) in *cJUN* knockout (red) and wildtype (black) polyps. **(A)** Kiloniella (Alphaproteobacteria), **(B)** Mf105b01 (Alphaproteobacteria), **(C)** Rhizobiales (Alphaproteobacteria), and **(D)** Alcanivorax (Gammaproteobacteria) show distinct colonization patterns between genotypes. Significant differences between groups at specific time points are indicated (*p<0.05, **p<0.01, ***p<0.001; ANCOM).

Discussion

Nematosomes exhibit characteristics of ancient immune cells

Our study provides strong evidence that nematosomes are key immune cells in *N. vectensis*, playing a critical role in microbial selection through selective phagocytosis. The phagocytosis assay demonstrated that nematosomes exhibit a clear preference for engulfing foreign *Vibrio* isolates while sparing native ones, indicating that the distinguishing of bacteria acts most likely on the level of recognition. Phagocytosis is a fundamental immune defense mechanism in invertebrates, often mediated by circulating cells called immunocytes. These cells fulfill various functions of the innate immune system, including the recognition of pathogens, phagocytosis and the synthesis of antimicrobial proteins. Thereby, the innate response triggered by microbial-associated molecular patterns (MAMPs) is based on the activation of pattern recognition receptors. In vertebrates, macrophages use pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and scavenger receptors to recognize MAMPs and discriminate between self and non-self by activating NF-κB (61, 62). In nematosomes of N. vectensis the TLR signaling pathway is also highly expressed and has been shown to activate the NF-κB pathway in response to bacterial pathogens, mirroring the innate immune responses seen in vertebrates (29, 63). Experimental MyD88 knockdowns in *Hydra* showed that TLR signaling does not act unidirectionally via the transcription factor NF-κB but is also linked to cJUN, resulting in altered microbiome composition and impaired bacterial recognition (58). This suggests that nematosomes may use TLR-mediated pathways to recognize bacterial cells and regulate strain-specific immune responses through evolutionarily conserved signaling cascades.

Furthermore, the observed correlation between phagocytosis efficiency and bacterial colonization efficiency supports the hypothesis that nematosomes act as selective gatekeepers of microbial establishment in *N. vectensis*. Native *Vibrio* isolates that were less frequently phagocytosed colonized the polyps at higher rates, while foreign isolates that were preferentially engulfed and degraded exhibited poor colonization efficiency. The colonization by foreign isolates increased in *cJUN*$^{-/-}$ polyps, demonstrating that nematosomes play a direct role in shaping the microbiome by selectively removing non-native bacteria before they can establish themselves in the host. The proteomic analysis

further supports this immune function, revealing distinct protein patterns in nematosomes upon bacterial exposure. Proteins involved in phagocytosis, including cytoskeletal components such as actin and dynein, were upregulated in response to foreign bacteria, supporting the notion that nematosomes are actively involved in immune surveillance and microbial selection, and emphasizing the functional importance of nematosomes in host-microbe interactions.

cJUN - a regulator of nematosome phagocytosis and proliferation

Our results highlight cJUN as a crucial regulator of innate immunity in *N. vectensis.* CRISPR/Cas9-mediated knockout of *cJUN* resulted in a marked impairment in their ability to mount a selective immune response. In *cJUN*$^{-/-}$ mutants, nematosomes exhibited a failure to activate lysosomal degradation pathways following bacterial engulfment, leading to an accumulation of both native and foreign bacteria within the phagosome. Similarly, macrophages, disrupted in *cJUN*, TLRs, or NF-κB, can still engulf pathogens but fail to complete their degradation (64, 65, 66), resulting in “arrested phagocytosis”, where bacteria remain trapped within the phagosomes but are not effectively digested. Certain intracellular pathogens, exploit these host weaknesses by preventing phagosome-lysosome fusion or modifying host signaling pathways to survive within immune cells (67, 68, 69). These evasion tactics allow pathogens to manipulate key stages of the phagocytosis process, including phagosome formation, maturation, and acidification (69). By interfering with these crucial immune defense mechanisms, bacteria can avoid degradation and establish intracellular infections. However, also symbionts rely on the mechanisms of arrested phagocytosis to persist within host tissue. In sponges, ankyrin-repeat proteins from bacterial symbionts can modulate phagocytosis by interfering with phagosome development, potentially allowing symbionts to escape digestion (70, 71). Similarly, in deep-sea mussels, the regulation of mTORC1 signaling helps retain symbionts in gill cells by preventing phagosome digestion (72). In coral-dinoflagellate symbiosis, the symbiosome is hypothesized to be an early arrested phagosome, with transient gene expression changes occurring during symbiont uptake (73). Recent results indicate, that anthozoan hosts indiscriminately phagocytose various microalgae, but non-symbiotic species are expelled through vomocytosis. Successful symbionts suppress the

host's innate immune response, preventing expulsion and promoting niche formation (74). These studies highlight the importance of arrested phagocytosis in various symbiotic relationships across different marine organisms.

In *N. vectensis cJUN*$^{-/-}$ nematosomes exhibited a similar phenotype, where they successfully internalized bacteria but failed to activate lysosomal responses necessary for degradation. The accumulation of engulfed but undegraded bacteria in mutant nematosomes suggests that cJUN plays a crucial role in regulating lysosomal maturation and phagosomal acidification. This parallels its function in vertebrates, where *cJUN* is involved in the transcriptional regulation of immune effectors, including lysosomal enzymes and phagosome maturation factors (59). The absence in cJUN-dependent signaling in *N. vectensis* may thus impair the degradation of foreign *Vibrio* strains, contributing to microbial persistence and altered colonization patterns. This suggests that cJUN is essential for orchestrating the cytoskeletal and phagosomal dynamics required for effective microbial clearance.

In addition, cJUN regulates also nematosome proliferation, as demonstrated by the significant reduction in nematosome numbers in *cJUN*$^{-/-}$ mutants. It is well known that cJUN positively regulates cell proliferation by repressing tumor suppressor genes and inducing cyclin D1 transcription in invertebrates (75, 76) and vertebrates (56). Thereby, cJUN negatively regulates p53 expression by binding to its promoter, thereby promoting cell cycle progression and proliferation (77). Nematosomes are budding from distinct regions of the mesenteries into the gastric cavity. In addition to the proliferation of nematosomes, the mesenteries of *N. vectensis* play a crucial role in endomesodermal patterning and germ cell development (78, 79), demonstrating the highly proliferative properties of this tissue. Our results show that cJUN is essential for controlling the expansion of nematosomes, potentially by regulating genes involved in cell division and differentiation, suggesting that cJUN not only governs phagocytosis but also orchestrates the development and maintenance of nematosomes as functional immune units.

The loss of *cJUN* function ultimately resulted in microbial dysbiosis, with mutant polyps displaying altered microbiome compositions dominated by non-native bacterial species. These findings establish a direct link between cJUN-mediated immune regulation and microbiome homeostasis in *N. vectensis*. The transcription factor cJUN plays a crucial role in immune regulation and homeostasis. In *Drosophila*, JNK signaling, which activates

cJun, is essential for innate immunity and development (80). In mammals, epidermal JunB regulates cutaneous immune cell-microbiota interactions, with its absence leading to atopic dermatitis-like symptoms and spontaneous S. aureus colonization (81). cJun/AP-1 is particularly important in CD8 T cell responses to acute infection, participating in productive immune responses (82). These studies highlight the complex interplay between c-Jun-mediated signaling, immune regulation, and microbiome homeostasis in various organisms and contexts.

Innate immune specificity and its implications for host-microbe interactions

The strong evidence for innate immune specificity in *N. vectensis* has significant implications for our understanding of the evolution of host-microbe interactions. Traditionally, innate immunity has been viewed as a broad, non-specific defense mechanism, whereas adaptive immunity is considered the primary driver of immune specificity. However, our findings challenge this dichotomy by demonstrating that even early-branching metazoans like cnidarians possess selective innate immune mechanisms. The ability of nematosomes to distinguish between closely related bacterial strains and selectively regulate microbiome composition suggests that innate immune specificity is an ancient and fundamental feature of metazoan immunity. This aligns with recent studies in other invertebrates, which have also demonstrated a surprising degree of innate immune selectivity, further supporting the notion that immune specificity predates the evolution of adaptive immunity (83, 84). In several studies it was shown that invertebrates can differentiate between pathogens at the species and even strain level (85, 86). This specificity is particularly evident in the phenomenon of immune priming, where initial exposure to a pathogen provides protection against subsequent encounters. Immune priming in invertebrates is a phenomenon where an initial pathogenic exposure enhances immune defenses against subsequent infections. This adaptive-like immunity has been observed in various invertebrates (87, 88), such as in woodlice (8), where hemocytes show increased phagocytosis of previously encountered bacterial strains. In the oyster *Crassostrea gigas*, hemocytes exhibit differential phagocytic responses to various bacterial species, demonstrating that invertebrate immune cells can selectively

recognize and respond to different microbes (89, 90). Similarly, in the squid-*Vibrio* symbiosis, host immune cells differentiate between preferred symbionts and other closely related bacteria (16). In addition, immune priming can be enhanced by protective symbionts (91). Potential mechanisms involved are a sustained immune responses, epigenetic modifications, and metabolic reprogramming, though the underlying mechanisms are not fully understood (92). However, C-type lectin-like domain (CTLD) proteins have been identified as potential contributors to this specificity, with their extreme gene diversification observed in various invertebrate genomes (93). *Drosophil*a exhibits a specific primed immune response against certain pathogens based on phagocytosis (94) that requires phagocytes and the Toll pathway. This priming involves exposure to dead or sublethal doses of microbes, eliciting an initial response that enhances protection against subsequent infections (95) . While the mechanisms underlying this specificity and memory are not fully understood, proposed explanations include elevated levels of phagocytosis. These examples provide additional support for the concept that innate immune specificity is an ancient and widespread phenomenon across diverse metazoans.

The evolutionary advantage of innate immune specificity likely lies in its ability to balance microbial diversity while preventing colonization by potentially harmful bacteria. In the case of *N. vectensis*, the selective phagocytosis of foreign bacteria by nematosomes ensures that the microbiome remains stable and beneficial to the host. This mechanism is particularly crucial for organisms with simple immune architectures, where adaptive immune responses are absent. By employing a finely tuned innate immune response*, N. vectensis* can maintain a dynamic but controlled microbiome, allowing for environmental adaptability without compromising immune defenses. This study, therefore, positions cnidarians as valuable models for exploring the evolutionary origins of immune-microbe interactions and provides insights into how early metazoans may have developed mechanisms for microbial regulation in the absence of adaptive immunity.

Conclusion

In conclusion, our study provides compelling evidence that *N. vectensis* employs a selective innate immune system to regulate its microbiome, challenging the traditional perception of innate immunity as a non-specific defense. The role of nematosomes in selectively phagocytosing foreign bacteria, and the involvement of cJUN in orchestrating this process, highlights the molecular complexity of immune regulation in early metazoans. Our findings reinforce the idea that innate immune specificity is evolutionarily ancient and widespread among invertebrates, playing a crucial role in maintaining host-microbe homeostasis. By demonstrating the impact of selective immune responses on microbiome composition, our work contributes to a broader understanding of host-microbe interactions and their evolutionary significance. Future research should further explore the molecular pathways underlying nematosome-mediated immunity and examine how these mechanisms have influenced the evolution of immune systems across metazoans.

Supplement Material

Table 2-S1 Enriched KEGG pathways across protein clusters identified in Nematosomes from Nematostella vectensis*. Each row represents a KEGG pathway significantly enriched within a specific protein cluster. The table includes KEGG pathway identifier and name ("Term"), number of genes in the input list associated with the pathway ("Count"), percentage of the cluster they represent ("%"), enrichment p-values ("p-value"), gene identifiers ("Genes"), and pathway enrichment statistics including the total number of genes in the list ("List Total"), the number of genes from the background that hit the pathway ("Pop Hits"), the total number of background genes ("Pop Total"), and the calculated fold enrichment ("Fold Enrichment").*

Cluster	Term	Count	%	p-value	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment
1	nve00190: Oxidative phosphorylation	10	6.37	2.4E-06	A7RJL7, A7SX99, A7RRK0, A7SCJ3, A7S0D1, A7RI03, A7RZ03, A7SPP0, A7SD20, A7SK19	64	72	3714	8.06
1	nve01200: Carbon metabolism	7	4.46	5.6E-03	A7RHJ6, A7RI62, A7SRY1, A7SCJ3, A7S6F3, A7RPB4, A7RT86	64	97	3714	4.19
1	nve00910: Nitrogen metabolism	3	1.91	7.4E-03	A7S717, A7S6F3, A7S762	64	8	3714	21.76
2	nve00250: Alanine, aspartate and glutamate metabolism	3	3.00	1.6E-02	A7SPW2, A7RT61, A7SXV0	27	28	3714	14.74
3	nve00280: Valine, leucine and isoleucine degradation	4	3.33	8.0E-03	A7RFT6, A7SF39, A7RVM2, A7SFM8	38	42	3714	9.31
3	nve00640: Propanoate metabolism	3	2.50	3.1E-02	A7RFT6, A7SF39, A7SLY9	38	28	3714	10.47
3	nve01240: Biosynthesis of cofactors	5	4.17	3.3E-02	A7RWF3, A7RFT6, A7RVA9, A7SFM8, A7RG51	38	123	3714	3.97
3	nve01100: Metabolic pathways	18	15.00	3.3E-02	A7T0R9, A7SY95, A7RU24, A7RVM2, A7SCI4, A7RVA9, A7SFM8, A7T002, A7RT53,	38	1130	3714	1.56

					A7RWF3, A7RFT6, A7SEM3, A7SF39, A7S4Z2, A7SLY9, A7SNG6, A7SD62, A7RG51				
3	nve00190: Oxidative phosphorylation	4	3.33	3.4E-02	A7SNY2, A7RU24, A7S4Z2, A7RT53	38	72	3714	5.43
3	nve00020: Citrate cycle (TCA cycle)	3	2.50	3.5E-02	A7RU24, A7RFT6, A7SLY9	38	30	3714	9.77
3	nve00670: One carbon pool by folate	3	2.50	4.9E-02	A7SY95, A7RFT6, A7RG51	38	36	3714	8.14
4	nve01230: Biosynthesis of amino acids	6	5.77	4.5E-04	A7RYI9, A7SEP6, A7RZV8, A7RN43, A7RMR5, A7S2F7	39	65	3714	8.79
4	nve00270: Cysteine and methionine metabolism	4	3.85	9.8E-03	A7RYI9, A7SEP6, A7RQE0, A7RMR5	39	44	3714	8.66
4	nve04145: Phagosome	5	4.81	1.2E-02	A7SJX9, A7S1V0, A7T9Q8, A7SAS6, A7RRH9	39	88	3714	5.41
4	nve00620: Pyruvate metabolism	3	2.88	4.2E-02	A7SRZ0, A7RL06, A7S2B4	39	32	3714	8.93
4	nve01100: Metabolic pathways	18	17.31	4.3E-02	A7S0P8, A7SU04, A7RN43, A7RHR8, A7RMR5, A7SDH5, A7SFK7, A7RL06, A7RKY4, A7SRZ0, A7RYI9, A7SEP6, A7RZV8, A7RQE0, A7SA87, A7S2F7, A7SAS6, A7S2B4	39	1130	3714	1.52
5	nve03010: Ribosome	33	12.36	8.0E-19	A7SU78, A7S4S4, A7S786, A7SUJ1, A7SGN4, A7SN77, A7SBD1, A7T1P0, A7SQP1, A7RUW3, A7SRV8,	151	121	3714	6.71

					A7RL80, A7RI41, A7T0C1, A7S2J5, A7RLF6, A7RSF0, A7S3J7, A7REW3, A7RYT2, A7RJJ7, A7S5T9, A7RS93, A7RFJ1, A7S494, A7SDI8, A7RGT2, A7SAM6, A7S072, A7S3C2, A7SH82, A7SHY5				
5	nve00280: Valine, leucine and isoleucine degradation	8	3.00	1.2E-03	A7SWC2, A7SLW1, A7SEN0, A7RGP1, A7RHX8, A7SJI9, A7SHB8, A7RQC1	151	42	3714	4.68
5	nve01230: Biosynthesis of amino acids	8	3.00	1.5E-02	A7SLW1, A7SFV8, A7SF13, A7RU37, A7RER9, A7S2W5, A7S143, A7RIZ1	151	65	3714	3.03
5	nve03050: Proteasome	6	2.25	1.7E-02	A7SQQ7, A7T284, A7SYY2, A7SZN8, A7RGH4, A7SIL7	151	38	3714	3.88
5	nve00190: Oxidative phosphorylation	8	3.00	2.5E-02	A7SQT8, A7RV43, A7S1B9, A7RG58, A7RNI6, A7RL79, A7SUW1, A7T224	151	72	3714	2.73
5	nve01100: Metabolic pathways	58	21.72	2.6E-02	A7S4Q7, A7RG06, A7RIM3, A7SR35, A7S1S6, A7SEN0, A7S1B9, A7SBT1, A7SXK7, A7RX31, A7S0R9, A8DWA9, A7S338, A7SJI9, A7SPM9, A7RER9, A7RL79, A7SUW1, A7SR87,	151	1130	3714	1.26

					A7S773, A7T224, A7RQV8, A7SFU6, A7SQB8, A7SJQ0, A7RHX8, A7RG58, A7RMA1, A7RIZ1, A7SKB9, A7RH72, A7SQT8, A7RV43, A7RNI1, A7RNI6, A7S382, A7S2W5, A7S143, A7SJJ1, A7SWC2, A7SFR2, A7RIW3, A7SFV8, A7SF13, A7SYK1, A7SD96, A7RIH5, A7RU37, A7SY83, A7S6X6, A7SLW1, A7RET7, A7RGP1, A7SG03, A7S392, A7SHB8, A7RQC1, A7REX4				
5	nve00270: Cysteine and methionine metabolism	6	2.25	3.1E-02	A7SLW1, A7SFV8, A7SQB8, A7S143, A7SJJ1, A7RH72	151	44	3714	3.35
5	nve01200: Carbon metabolism	9	3.37	4.1E-02	A7RX31, A7SFV8, A7SF13, A7RGP1, A7RU37, A7RER9, A7SYK1, A7RIZ1, A7SR35	151	97	3714	2.28
	nve03010: Ribosome	10	13.16	2.5E-07	A7RLY2, A7SK96, A7RLM9, A7S405, A7SNU0, A7RKV4, A7SUQ5, A7SES9, A7SKY1, A7SCX9	31	121	3714	9.90

Author contribution

NK, and SF conceptualized the study. NK performed the experiments and did the interpretation. MA did the proteomic sequencing (Figure 2-3, 2-4, Table 2-S1) and wrote the “LC-MS Proteomics and Data analysis” part in the Methods, NK completed the sample preparation. GF did wildtype mono-association experiment (Figure 2-2E, F). NK wrote the first draft of the manuscript. SF, UH and MA contributed to manuscript revision.

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Chapter 3

Microbiome-induced innate immune training in the sea anemone *Nematostella vectensis*

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Abstract

Trained immunity, the capacity of innate immune cells to respond differently to future microbial challenges based on past encounters, is emerging as a conserved phenomenon across animal phyla. However, direct evidence for such memory-like behavior in early-diverging animals remains limited. Here, we demonstrate that the migratory immune structures known as nematosomes in the sea anemone *Nematostella vectensis* undergo persistent, strain-specific functional reprogramming following microbial exposure. Using a germfree recolonization approach with defined native symbionts, we show that nematosomes exhibit reduced phagocytic and lysosomal responses to homologous bacterial strains upon re-exposure, indicative of functional immune tolerance. In contrast, responsiveness to heterologous strains remains intact, suggesting a trained immunity phenotype characterized by selective restraint. These effects are abolished in *cJUN* knockout animals, implicating a conserved transcriptional regulator in the modulation of memory-like immune behavior. Our findings demonstrate that immune plasticity in *Nematostella* is shaped by microbial identity, can lead to both training and tolerance, and is governed by transcriptional reprogramming. This study establishes nematosomes as an invertebrate model for innate immune training and provides new insights into the evolutionary origins of immune memory.

Introduction

Host–microbe interactions are fundamental to animal health, development, and immune function across the metazoan tree of life (1). In particular, microbial communities play a crucial role in shaping immune responses, influencing pathogen defense, immune homeostasis, and even developmental outcomes (2, 3, 4). In marine invertebrates, the microbiome is intricately linked to processes including development, defense mechanism, and environmental adaptation such as thermal adaptation (5, 6, 7, 8, 9).

Innate immunity is classically defined as a rapid, broad-acting defense system, whereas adaptive immunity offers slower but highly specific responses with long-term memory (10). A key feature of innate defense is phagocytosis, a process deeply conserved across animal phyla. It enables immune cells to recognize, internalize, and degrade foreign particles or altered self, relying on a coordinated series of steps from target recognition to phagolysosome maturation (11, 12, 13, 14, 15). Invertebrate phagocytes display striking mechanistic parallels to vertebrate counterparts, including cytoskeletal dynamics and energy dependence (16). Notably, in filter feeding animals enteric phagocytes also function in nutrient digestion, reflecting dual roles in immunity and nutrition (17). Lysosomes serve as a central hub in these processes, acting as both degradative and signaling organelles essential for effective immune responses (18, 19).

Recent findings on host-microbe interactions suggest that innate immune systems, which were previously considered to be rigid and non-specific, exhibit a surprising degree of plasticity. These findings include the concept of trained innate immunity (20, 21), defined as a form of innate immune memory that modifies the manner in which organisms respond to subsequent microbial encounters. Innate immune training, originally described in vertebrate myeloid cells and macrophages, is hypothesized as an evolutionarily conserved form of memory-like adaptation present across diverse animal phyla, including invertebrates (20, 22, 23, 24). Unlike adaptive immunity, which relies on antigen-specific receptors and lymphocyte memory, trained innate immunity involves long-term modulation of innate effector functions through epigenetic, metabolic, or transcriptional changes (22, 25). In vertebrates, trained innate immunity was first observed in monocytes and macrophages, where prior exposure to pathogens leads to enhanced responses upon reinfection (24, 26). This phenomenon, marked by improved microbial clearance, is now

recognized in invertebrates as well (25, 27). In insects, for example, a process known as 'immune priming' in invertebrates enhances phagocytic activity and promotes survival upon reinfection. Also, crustaceans and mollusks exhibit distinct immune responses tailored to previously encountered microbial strains (28, 29). These findings challenge the long-held notion that innate immunity is strictly short-lived and non-specific, instead revealing a surprising degree of plasticity and memory-like behavior even in basal metazoans.

This capacity for enhanced responsiveness is complemented by the equally important mechanism of immune tolerance, which terminates the response to foreign antigens and maintains insensitivity to self-antigens (30). The microbiota plays a crucial role in shaping and maintaining this immune tolerance while protecting against pathogens (31, 32). In vertebrates, immune tolerance is enforced by regulatory T cells, inhibitory receptors, and cytokine-mediated immune suppression (33, 34, 35). In Drosophila, tolerance is achieved through selective antimicrobial peptides (AMP) expression regulated by proteins like PIMS, and p38 MAPK-dependent phagocytic encapsulation of bacteria (36, 37).

Microbial discrimination mechanisms preserve beneficial symbioses while limiting overactivation of immune responses. For instance, TGF-β pathways in cnidarians may promote tolerance of dinoflagellate symbionts (38). Thus, we hypothesis that immune training and tolerance represent two complementary outcomes of host–microbe interactions: the former enhances future defense, while the latter restrains immune responses. Therefore, both processes may reflect the dynamic and context-dependent nature of innate immunity in early-diverging animals.

To test this hypothesis, we use the marine model organisms *Nematostella vectensis,* which has a relatively simple body plan and tractable microbiome. The innate immune system of *Nematostella* is characterized by conserved NF-κB and TLR signaling (39, 40), which are also expressed in nematosomes. Nematosomes are migratory cell clusters specific to *Nematostella* and have recently been identified as immune effector units capable of selective phagocytosis ((17); Chapter 2). We could show, that cJUN is an important transcriptional regulator for nematosome function, including its role in lysosomal maturation. Furthermore, cJUN is involved in microbiome regulation by phagocytosing foreign bacteria and tolerating native colonizers.

However, whether nematosomes can be trained to tolerate specific bacterial colonizers by mechanisms of innate immune training remains unknown. Here, we examine how different microbial colonization modulates nematosome innate immune responses. Using germfree *Nematostella* polyps recolonized with defined bacterial symbiont strains (NJ1, NA68, and NA29), we tracked bacterial engulfment and lysosomal activity after homologues and heterologous exposure. In addition, we evaluated the role of cJUN in regulating these processes, given its established role in immune signaling and lysosomal function.

Our findings reveal that exposure to symbionts leads to strain-specific immune imprinting, reducing nematosomal reactivity upon re-encounter with the same bacterial strain, while maintaining increased reactivity to heterologous strains. This trained tolerance was strain specific and required the transcription factor cJUN. Taken together, our results support the existence of trained innate immune tolerance in *Nematostella vectensis*, indicating that basal metazoans can develop sustained, microbe-informed immune modulation shaped by prior encounters.

Methods

Nematostella culture management

Adult *Nematostella vectensis* cultures were derived from F1 offspring originating from CH2XCH6 parental lines collected from the Rhode River in Maryland, USA. Animals were maintained in Nematostella Medium (NM) adjusted to a salinity of 16‰ using Red Sea Salt®. Cultures were housed in separate containers under dark conditions at a constant temperature of 18°C. Animals were fed daily with *Artemia salina* larvae and received weekly media changes with fresh NM.

Generation of germfree polyps

Germfree *Nematostella* polyps were generated by adapting the antibiotic protocol described by Domin et al. (41) for *Hydra* polyps (42). Polyps were treated for two weeks with a combination of antibiotics (Ampicillin, Streptomycin, Neomycin, Spectinomycin, Rifampicin; 50 µg/ml each) dissolved in sterile-filtered 16‰ NM. The antibiotic medium was refreshed daily, and polyps were transferred to sterile containers every two days. Following antibiotic exposure, polyps underwent a recovery phase of three days in antibiotic-free sterile NM. Control polyps (wildtype, WT) were maintained in sterile NM without antibiotics. All polyps were kept unfed in darkness at 18°C throughout the treatment. Sterility of polyp's post-antibiotic treatment was assessed by homogenizing individual polyps onto Marine Bouillon (MB) agar plates to detect cultivable bacterial growth. Plates were incubated at room temperature, and absence of bacterial colonies after 48 hours confirmed successful germfree status.

Recolonization and extracting nematosomes

To investigate the hypothesis that bacterial colonization state affects the lysosomal activity of nematosomes, conventionalization and recolonization experiments were performed. Experimental groups included wildtype (WT), conventionalized (Conv), mono-association (Mono), and germfree (GF) polyps. GF, Rec, and Conv groups underwent the antibiotic treatment described above, whereas WT polyps were maintained in sterile NM without antibiotics for two weeks at 18°C in darkness without feeding. Conv polyps were inoculated with microbiome solutions prepared from homogenizing five WT animals in

sterile-filtered NM, distributing 4 ml of this microbiome suspension per well. Polyps were incubated for one week under these conditions to facilitate microbiome establishment. The mono-association group was exposed specifically to native bacteria strains (NJ, NA68 and NA29). Bacteria were cultured overnight in Marine Broth (MB) at 30°C until exponential growth phase (OD600 <0.1). After additional incubation at 30°C (1-2 hours), bacterial cultures were adjusted to an optical density (OD600) of 0.1. Polyps were recolonized by exposure to this bacterial culture for one week.

Nematosomes were extracted following the conventionalization and mono-association experiments. Polyps were placed under sterile conditions in a petri dishes, and nematosomes were manually extracted from the foot region using a scalpel under a stereomicroscope without killing the polyps. Extracted nematosomes were transferred to chamber slides (Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ System) for staining. Staining was conducted using Hoechst (10nM) dye to visualize DNA in nuclei and Lysotracker (15nM) to identify lysosomal activity. Hoechst dye binds specifically to nuclear DNA, emitting blue fluorescence, while Lysotracker selectively labels acidic lysosomal compartments, emitting red fluorescence.

Assessment of phagocytic activity after re-exposure with bacteria in nematosomes

Following one week of colonization, extracted nematosomes were challenged again with their corresponding bacterial strains, previously stained with BacLight dye (Thermo Fisher) to distinguish live and dead bacterial cells. Nematosomes were incubated with stained bacteria for 90 minutes at room temperature. Post bacterial challenge, nematosomes were stained with Hoechst and Lysotracker for 45 minutes, rinsed in PBS, and fixed using 3% paraformaldehyde (PFA). Samples were mounted using fluorescent-compatible ProLong™ Diamond (Thermo Fisher) and imaged by fluorescence microscopy. Fluorescence was visualized at wavelengths of 516 nm (BacLight), 668 nm (Lysotracker), and 461 nm (Hoechst). Images were processed using ImageJ software (43). After adjusting contrast and converting images to 8-bit format, fluorescence intensity thresholds were set, and lysosomal areas relative to nematosome size were quantified and expressed as percentages. Statistical analyses were conducted using Origin Pro software (Version 2021. OriginLab Corporation, Northampton, MA, USA.).

Phylogenetic analysis

16S rRNA gene sequences of bacterial isolates (NJ1, NA68, and NA29) (44) were PCR-amplified and Sanger-sequenced using universal primers 27F and 1492R. Resulting sequences were quality-trimmed and aligned against the NCBI nucleotide database using BLAST to identify closest relatives. To place the isolates in a phylogenetic context, we selected full-length or near-full-length 16S rRNA reference sequences from closely related *Vibrio* species and other marine-associated bacteria, including *Vibrio fischeri* (FJ464360.1) and *Vibrio coralliilyticus* (JN039154.1). Sequences were aligned using ClustalW in MEGA (Version 11.0.13) (45) and a maximum likelihood phylogenetic tree was constructed using the Tamura-Nei substitution model (46). Bootstrap analysis was performed with 100 replicates to assess node support. The final tree was visualized and annotated in MEGA and exported in TIFF format for downstream figure preparation.

Results

Microbial colonization alters lysosomal activity in nematosomes

To investigate whether microbial colonization modulates innate immune activity in *Nematostella vectensis*, we analyzed lysosomal activity in nematosomes of polyps in regard to microbial colonization. In the context of immune response, enhanced lysosomal function is often used as a proxy for heightened immune potential following microbial exposure (47, 48). We treated polyps with antibiotics and colonized them with a complex community (conventionalized). After two weeks of antibiotic treatment and 7 days of recolonization, nematosomes were extracted and lysosomal activity determined. We compared the lysosomal activity of nematosomes of the recolonized polyps (Conv) with that of germfree (GF) and wildtype (WT) control polyps (**Figure 3-1A, B**). Germfree polyps exhibit significantly reduced lysosomal activity compared to WT polyps. This reduced lysosomal level was increased in conventionalized polyps to a similar level as in wt polyps. The reduced lysosomal signal in germfree animals indicate that nematosomes require microbial cues to maintain an active immune state, and that this activation can be reinitiated upon bacterial exposure. These results demonstrate that nematosomes not only respond dynamically to microbial input but also appear capable of immune resetting, a key prerequisite of trained immunity.

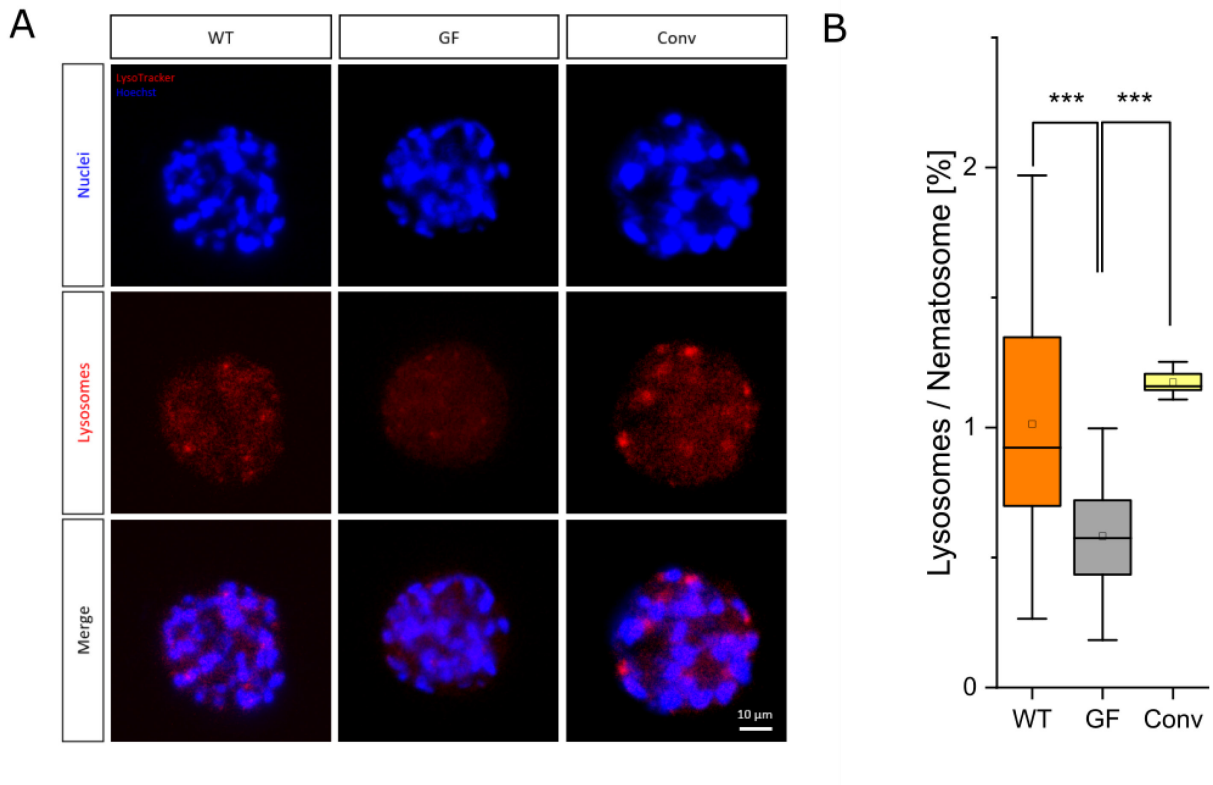

Figure 3-1 Lysosomal activity in nematosomes is modulated by microbial association. (A) Representative confocal images comparing lysosome content in nematosomes from wildtype (WT), germfree (GF), and conventionalized (Conv) animals. Nuclei are stained with Hoechst (blue), and lysosomes are marked using LysoTracker (red). Scale bar = 10 µm. **(B)** Quantification of lysosome content per nematosome across different conditions (WT, GF, Conv). Nematosomes from conventionalized animals showed significantly higher lysosome content compared to WT and GF conditions, indicative of microbiome-mediated immune modulation. Statistical significance was tested with One-way ANOVA, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (N = 20-120).

Prior symbiont colonization induces strains-specific immune training and tolerance in nematosomes

To evaluate whether nematosomes can be functionally trained by prior symbiont exposure, we investigated how bacterial colonization (or "training") influences the phagocytic activity of nematosomes to a secondary homologues and heterologous exposure. Therefore, germfree polyps were first recolonized by the bacterial isolates NA68, NJ1 and NA29 (**Table 3-1**). Although all three isolates fall within the *Vibrionales*, they occupy distinct positions in the phylogenetic tree (**Figure 3-2A**).

Table 3-1 Identification and closest type strain matches of *Vibrio* isolates from *Nematostella vectensis*. 16S rRNA gene sequences from five bacterial isolates were compared to type strain sequences using BLAST. Each isolate's host species, sequence length, GenBank accession number (if available), and closest type strain matches with corresponding sequence IDs and percent similarity are listed.

Isolate	Host	Sequence length with (GenBank accession number)	Closest type strains (with Sequence ID)	Similarity (%)
NJ1	*Nematostella vectensis*	1455 bp (PQ455196.1)	*Vibrio diazotrophicus* (114217.1) *Vibrio vulnificus* (NR_036888.1)	99% 99%
NJ68	*Nematostella vectensis*	855 bp	*Vibrio coralliilyticus* (NR_117892.1) *Vibrio tubiashii* (NR_026129.1) *Vibrio brasiliensis* (NR_117887.1)	97% 97% 97%
NA29	*Nematostella vectensis*	728 bp	*Vibrio alginolyticus* (NR_113781.1) *Vibrio parahaemolyticus* (NR_119058.1) *Vibrio campbellii* (NR_119050.1)	99% 99% 99%

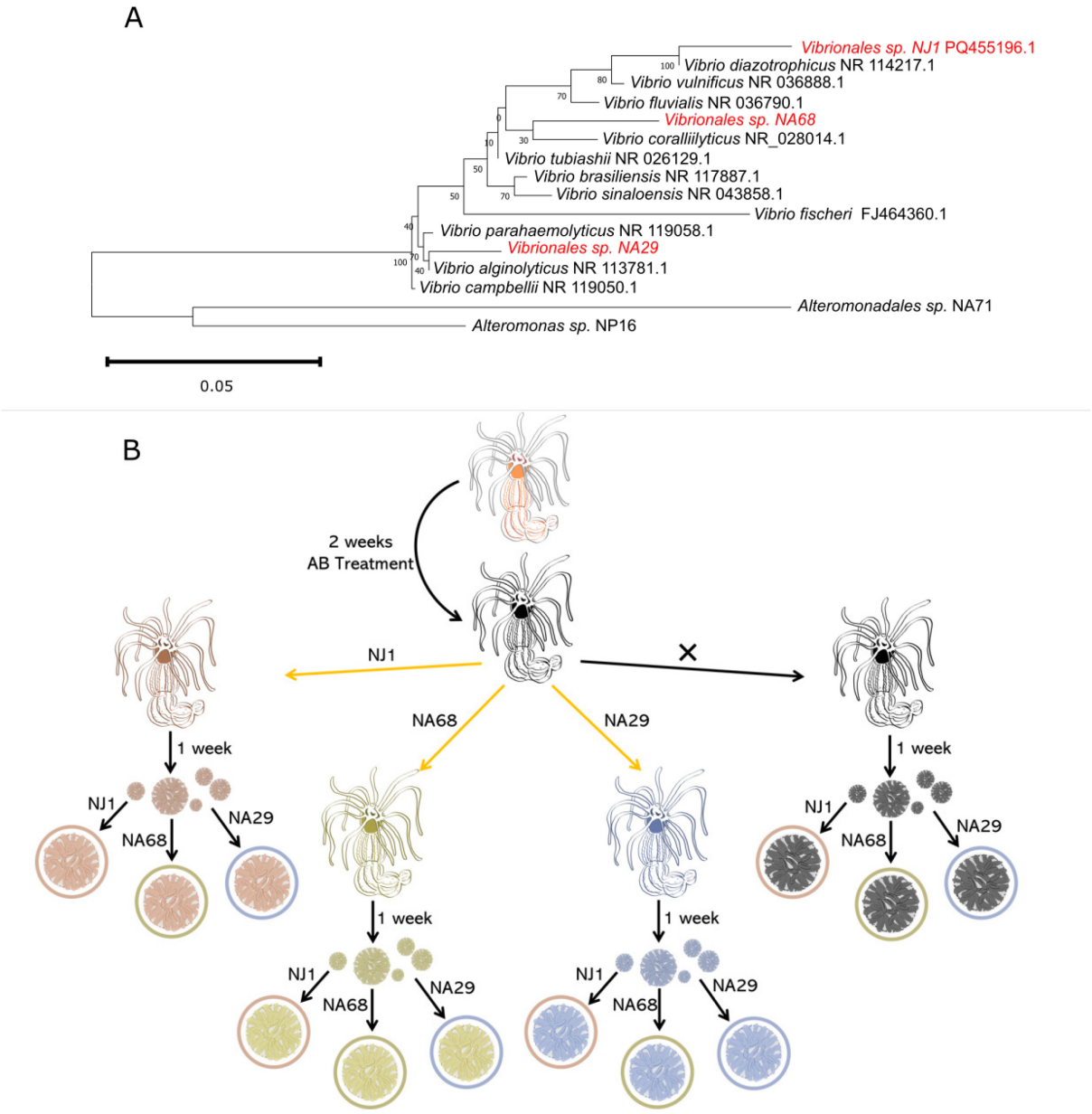

Figure 3-2 Phylogenetic placement and experimental design for bacterial training of *Nematostella vectensis*. (A) Maximum likelihood phylogenetic tree of 16S rRNA gene sequences showing relationships between isolated bacteria from *N. vectensis* (green) and selected reference strains. Scale bar indicates 0.05 substitutions per site. Strain names include accession numbers when available in brackets. **(B)** Schematic overview of the bacterial training experiment. Adult *Nematostella* polyps were treated with a cocktail of antibiotics (AB) for 2 weeks to deplete the native microbiota (middle, black polyps). These germfree animals were then recolonized for 1 week with one of three bacterial strains (NJ1, NA68, or NA29) and a group of germfree polyps remained germfree as the untrained group. After recolonization nematosomes were extracted and treated with the same isolates. Brown, green, and blue coloring denote the NJ1, NA68, and NA29 strains, respectively. The black arrow with an "X" indicates the absence of a challenge condition for germfree animals and represents the untrained group.

After one week of recolonization, nematosomes were isolated from these mono-colonized polyps and exposed to the three isolates reciprocally (**Figure 3-2B**). Untrained nematosomes were extracted nematosomes from former polyps which were not mono-associated with bacterial isolate and kept germfree. The nematosomes from these animals were also extracted after one week and treated with the same isolates as the other groups (**Figure 3-2B**). Quantification of phagocytosis rate and lysosomal activity revealed that nematosomes extracted from polyps exhibited a significant reduction in bacterial phagocytosis upon homologous exposure (trained) compared to not trained nematosomes (**Figure 3-3A-C**). This reduced phagocytosis was not observed in heterologous exposure, indicating that the training effect was strain-specific.

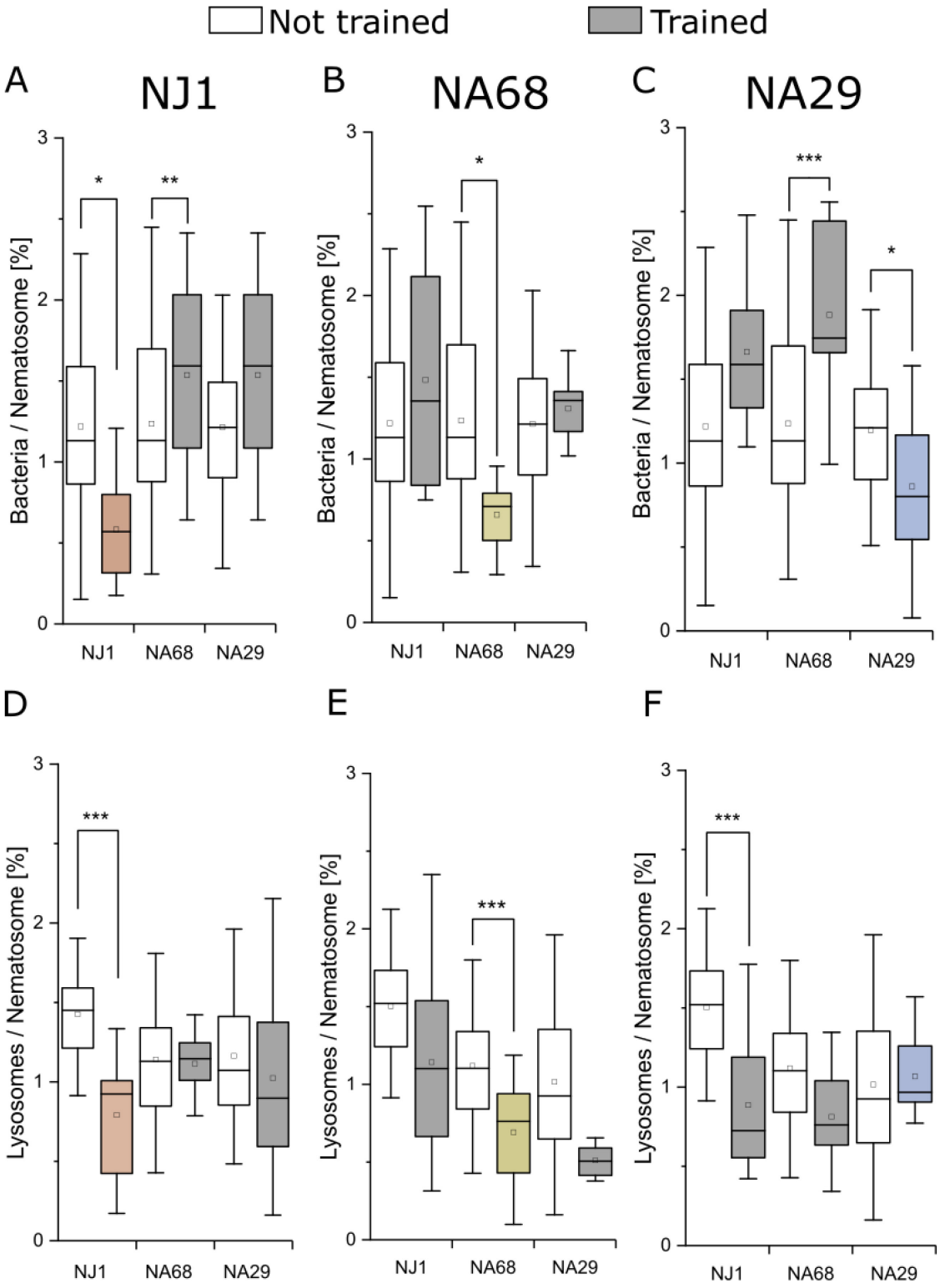

Figure 3-2 Strain specific training alters bacterial uptake and lysosomal activity in nematosomes. (A–C) Quantification of bacterial phagocytosis in nematosomes after training with strains NJ1, NA68, or NA29 and subsequent challenge with the indicated bacterial strain. Boxplots show the area of bacteria per nematosome (%). Trained nematosomes (dark gray) exhibit enhanced bacterial uptake in a strain-specific

manner compared to untrained controls (white). **(D–F)** Quantification of lysosomal content in the same nematosomes from panels A–C, showing strain-specific increases or decreases in lysosomal activity following bacterial training. Colored (brown, yellow, blue) boxes indicate homologous while grey boxes indicate heterologous bacterial re-exposure. All statistical significances were determined using one-way ANOVA with the following p-values: $p \leq 0.05$, $p \leq 0.01$, $*p \leq 0.001$, N=15-73.

Consistent with this, lysosomal activity showed that homologues exposure exhibited significantly lower activity compared to non-trained nematosomes in two cases. Only the homologues exposure to the strain NA29 did not lead to a reduced lysosomal activity (**Figure 3-2F**). In contrast, lysosomal activity to heterologous exposure lead not to a changed lysosomal activity compared to non-trained nematosomes (**Figure 3-2D-F**). This suggests that nematosomes adapt their phagocytic response based on prior microbial encounters, a key feature of innate immune training.

The transcriptional Factor cJUN regulates nematosome training dynamics

Given the emerging role of cJUN in innate immune regulation and lysosomal signaling, we tested whether this transcription factor is required for training-induced changes in nematosomal function. Our previous work established that *cJUN* is expressed in nematosomes and regulates their lysosomal machinery and phagocytic behavior (**Chapter 2**). To determine whether cJUN mediates innate immune training, we compared lysosomal activity in nematosomes from cJUN wildtype ($cJUN^{+/+}$) and knockout ($cJUN^{-/-}$) animals across different microbial treatments.

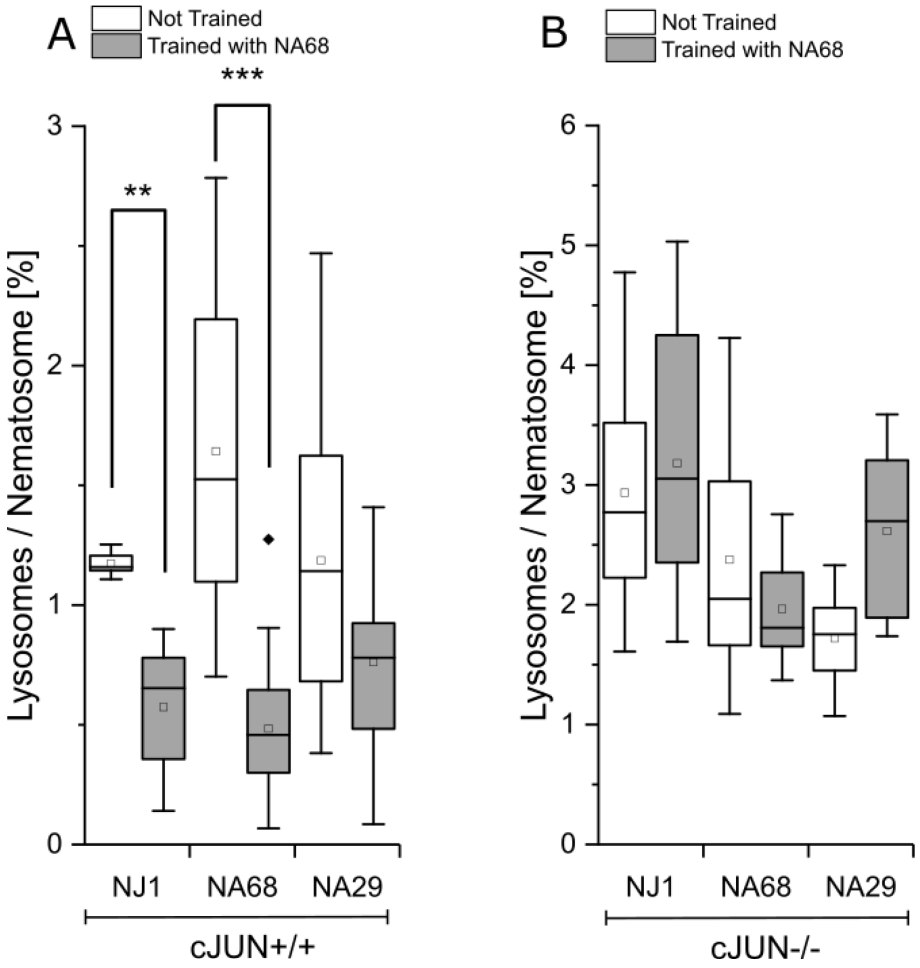

Figure 3-3 cJUN is required for strain-specific regulation of lysosomal activity in nematosomes. (A) Lysosomal content in nematosomes from wildtype (*cJUN*$^{+/+}$) animals that were either untreated (white) or exposed to NA68 (gray), followed by challenge with NJ1, NA68, or NA29. Exposure to NA68 leads to a significant decrease in lysosomal content upon second bacterial challenge. **(B)** Lysosomal content in nematosomes from *cJUN* knockout (*cJUN*$^{-/-}$) animals under the same conditions. The differential lysosomal response observed in wildtype animals is absent in *cJUN*-deficient nematosomes.

To test whether cJUN is essential for immune training, we performed bacterial training experiments using NA68 in *cJUN*$^{+/+}$ and *cJUN*$^{-/-}$ animals (**Figure 3-3**). Unlike wildtype nematosomes, which showed reduced lysosomal activity upon NA68 re-exposure, *cJUN*$^{-/-}$ nematosomes failed to modulate their response following re-exposure and exhibited uniformly high lysosomal signals regardless of microbial history (**Figure 3-3A, B**). These results indicate that cJUN is required for the adaptive modulation of lysosomal function following microbial training. Together, these findings position cJUN as a critical transcriptional regulator of innate immune training in *Nematostella vectensis*. In its

absence, nematosomes lose their ability to fine-tune lysosomal activity in response to prior microbial encounters, underscoring the role of conserved immune signaling pathways in modulating innate memory.

Discussion

Microbial training drives functional reprogramming and tolerance in nematosomes

Here, we provide the first evidence that non-bilaterian immune cells, the nematosome, exhibits key hallmarks of innate immune training. Our findings reveal that prior microbial exposure induces persistent, strain-specific modulation of phagocytic activity and lysosomal signaling in nematosomes, establishing a form of immune memory-like behavior previously undocumented in basal metazoans animals. This work expands the concept of trained immunity to cnidarians and introduces nematosomes as a novel invertebrate model for dissecting immune plasticity.

Nematosomes are migratory immune structures capable of classical innate responses, including phagocytosis and lysosomal degradation (17, 49). Our prior work established their role in microbial recognition and immune signaling via the transcription factor cJUN (**Chapter 2**). In this work we demonstrate that these immune functions are not fixed but are instead modified by prior microbial encounters. Specifically, nematosomes trained with a bacterial strain exhibited significantly reduced phagocytic uptake and lysosomal activity upon homologous re-exposure—effects not observed with heterologous bacteria. The reduced response upon homologous exposure is consistent with a model of functional immune tolerance, in which immune effector activity is actively suppressed following repeated encounters with a specific, non-threatening stimulus (50). This mechanism may serve to limit unnecessary immune activation against commensal or previously recognized symbionts, thereby preserving tissue integrity and avoiding chronic inflammation (51). Such tolerance-like outcomes have been described in invertebrates like mussels, where hemocytes show attenuated responses upon repeated exposure to *Vibrio splendidus* (52).

In our case, nematosomes appear to integrate microbial history to shift from an active defensive posture to a regulated, restrained state in a strain-specific manner. This may reflect a broader immune strategy in basal animals to distinguish not only self from non-self, but also friend from foe within the non-self-category. At the molecular level, such immune tolerance may be mediated through shifts in lysosomal biogenesis, autophagic flux, or redirection of transcriptional programs regulating inflammatory outputs (53, 54). In

mammalian systems, immune tolerance is often associated with regulatory macrophage phenotypes, where signaling through NF-κB, mTOR, or epigenetic modifiers leads to dampened inflammatory responses (55). Similar principles could underlie the observed nematosomal responses, suggesting that mechanisms for attenuating immune activation after microbial recognition might predate the evolution of adaptive immunity. These findings invite broader exploration into how cellular metabolism, lysosomal dynamics, and transcriptional plasticity contribute to innate immune restraint in early-diverging animals.

The specificity and durability of this response align more closely with trained immunity, as defined in vertebrate systems, than with the broader concept of invertebrate immune priming. Notably, while homologous exposure appears to induce tolerance, the persistent responsiveness of nematosomes to heterologous bacteria underscores a core feature of trained immunity: the capacity to mount enhanced or distinct responses to unfamiliar microbial stimuli following prior exposure. This dichotomy between tolerance and enhanced responsiveness highlights the functional plasticity of trained innate systems. While immune priming in insects and crustaceans often manifests as enhanced survival or systemic activation, trained immunity entails long-lasting, stimulus-specific functional reprogramming at the cellular level (56, 57, 58). In mammals, such training is well-characterized in monocytes and macrophages, where exposure to pathogens or microbial components such as β-glucans or BCG vaccine induces epigenetic and metabolic reprogramming, leading to altered responses upon re-infection. These responses are marked by increased cytokine production, enhanced phagocytosis, and improved pathogen clearance, often persisting over extended periods (59). In invertebrates like insects such as Tenebrio molitor exhibits species-specific immune priming, with stronger responses to certain pathogens like Metarhizium anisopliae and Bacillus thuringienses (60). This priming can be long-lasting, providing prophylaxis against future infections (61). In Drosophila melanogaster, chronic infection with certain bacteria provides broad, non-specific protection against secondary infections, increasing both resistance and tolerance (62). These examples are often described as instances of “immune priming”, a term commonly used in invertebrate immunity to denote enhanced responses upon re-exposure (23, 63, 64, 65). However, immune priming is typically defined at the organismal level and does not always imply persistent cellular or molecular changes in immune

effectors. In contrast, innate immune training, as defined in vertebrate systems, refers to long-lasting functional reprogramming of innate immune cells that leads to altered responsiveness upon secondary challenges, often through epigenetic, metabolic, or transcriptional changes (66, 67, 68, 69).

Our use of defined bacterial strains and germfree recolonization models enabled us to distinguish between generalized immune activation and microbial identity–dependent reprogramming. In this sense, nematosomes offer a cellular platform for investigating how microbial experience is integrated into immune behavior. Furthermore, nematosomes from germfree polyps exhibited markedly diminished lysosomal activity, supporting the idea that microbial exposure is required to initiate or maintain an immune-competent state. Reintroduction of defined bacteria reactivated lysosomal activity, suggesting that nematosomes can undergo reversible reprogramming based on microbial context.

These findings echo a broader principle seen in humans, where early-life microbial exposure is essential for immune development. The “hygiene hypothesis” proposes that reduced microbial exposure in early life may contribute to the rise in allergies and autoimmune diseases (70). Recent research indicates that diverse microbial exposure, particularly from the environment and family members, is crucial for developing a well-regulated immune system (71, 72). This exposure is most critical during pregnancy and early infancy (73). Our findings suggest that this principle may extend deep into animal evolution, with even basal metazoans like *Nematostella* relying on microbial signals to establish immune competence.

Mechanistic basis of strain-specific trained immunity in basal metazoans

The immune training observed in nematosomes was clearly strain-specific (**Figure 3-2**). Distinct bacterial isolates elicited divergent lysosomal responses, even in the absence of overt pathogenicity. This underscores the ability of nematosomes to discriminate among microbes at the strain level, which is a feature more commonly attributed to adaptive immune systems (74). In prior work, we showed that nematosomes preferentially phagocytose foreign *Vibrio* strains while sparing native microbiota individuals, and that this discrimination is cJUN-dependent (**Chapter 2**). In *Nematostella*, we found that cJUN

is indispensable for microbial training: in its absence, nematosomes fail to modulate lysosomal activity upon secondary exposure and display abnormally high baseline activity (**Figure 3-3**). This indicates that cJUN is a core regulator of trained immunity in this system, linking microbial sensing to effector modulation. Such specificity has been reported in other systems. The squid-*Vibrio* symbiosis between *Euprymna scolopes* and *Vibrio fischeri* involves the host's innate immune system, particularly macrophage-like hemocytes, in selecting beneficial symbionts while excluding other (75, 76). Hemocytes from symbiont-colonized squid show differential gene expression and reduced binding to *V. fisheri* compared to other bacteria (77). The specificity of this relationship is mediated by conserved innate immune mechanisms, such as MAMP/PRR interactions, and symbiont-specific features like luminescence (78). In Hydra and sea anemones, microbial colonization is shaped by Toll-like receptor (TLR) signaling and downstream immune effectors (39, 79, 80). These parallels suggest that nematosomes may utilize similarly conserved pathways, where TLRs, NF-κB, and MAPK/cJUN signaling are likely to orchestrate immune recognition and lysosomal regulation.

The implications of these findings extend beyond cnidarians. The ability of nematosomes to integrate microbial exposure into altered immune behavior suggests that innate immune training may be an ancestral trait. This capacity for functional reprogramming, seen in animals lacking lymphocytes or somatic recombination, argues that immune memory-like phenomena emerged early in metazoan evolution. By balancing microbial tolerance with selective activation, training mechanisms may have enabled basal animals to fine-tune immune responses in dynamic microbial environments.

Conclusion

Our study reveals that nematosomes in *Nematostella vectensis* undergo strain-specific functional reprogramming following microbial colonization. Homologous bacterial re-exposure induces immune tolerance, while heterologous exposure maintains responsiveness—a hallmark of trained immunity. These effects depend on microbial identity and require the transcription factor cJUN. Together, our findings establish a conserved mechanism of innate immune training in a basal metazoan and provide new insights into the evolution of immune memory in the absence of adaptive immunity.

Author contribution

NK, and SF conceptualized the study. NK and TA performed the experiments. NK performed the data analysis and interpretation. NK wrote the first draft of the manuscript. SF contributed to manuscript revision.

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General Discussion

Understanding how early-diverging animals regulate their microbial partners is essential to uncovering the evolutionary origins of immune specificity, host control, and microbial homeostasis. In this thesis, I investigated the interplay between environmental inputs, microbial compositions, and innate immune regulation in the estuarine cnidarian *Nematostella vectensis*. With its tractable genome, robust experimental tools, and evolutionary position at the base of the animal tree, *Nematostella* provides a unique opportunity to study the foundational principles governing host-microbiome interactions.

Framed by the central approach of **Host mechanisms controlling bacterial colonization in the sea anemone *Nematostella vectensis***, this thesis explores how nutritional state modulates microbial composition, how innate immune structures such as nematosomes contribute to microbial recognition and response, and how prior microbial encounters influence subsequent immune outcomes.

Host nutritional status influences microbial stability

The first chapter revealed that *Nematostella* polyps actively modulate microbial community composition in response to changes in nutrient availability. Starvation led to a reduction in polyp surface area and a sharp decline in microbial diversity, with a dominance shift toward stress-tolerant taxa like Spirochaetaceae. In contrast, frequent feeding preserved microbial diversity and supported a dynamic restructuring of the microbial community, characterized by greater compositional turnover and the persistence of Vibrionaceae and other host-associated taxa. Notably, polyps fed with germfree *Artemia salina* nauplii exhibited an even greater increase in microbial load than those fed with conventional food, suggesting that feeding stimulates endogenous host processes that promote bacterial colonization (**Figure-D 1**).

This could include increased mucus secretion, modulation of host-derived antimicrobial peptides, or metabolic fluxes that provide nutritional substrates for microbial proliferation. For instance, in both cnidarians and vertebrates, stress-induced shifts in epithelial secretions are known to alter microbial habitat quality, including changes in mucin composition that selectively favor symbionts over opportunists (188, 189, 190, 191)

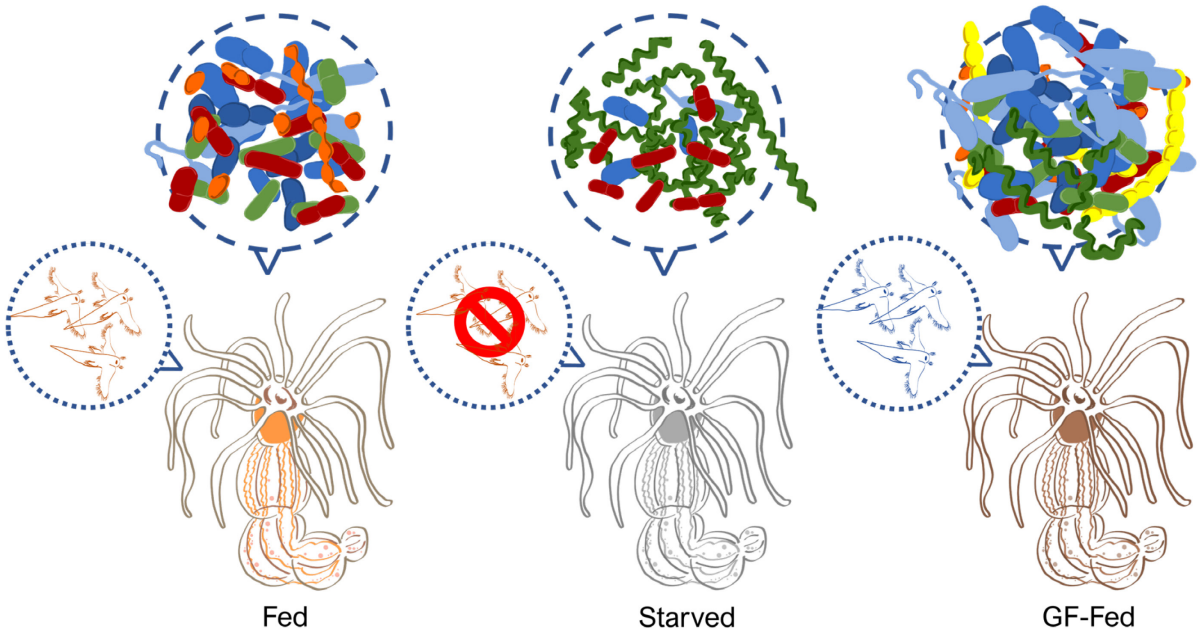

Figure-D 1 Nutritional status modulates microbial composition and proliferation in Nematostella vectensis. *Schematic representation of microbial dynamics in three experimental feeding conditions. In fed animals (left), a diverse and abundant microbiome is maintained, with active microbial proliferation. Starved*

animals (center) exhibit a loss of microbial diversity and dominance of stress-tolerant taxa (Spirochaetota), accompanied by marked reduction in microbial proliferation. In germfree (GF) Artemia- fed animals (right), microbial colonization is highly enhanced despite sterile food input, suggesting strong host-driven microbial recruitment and proliferation. These findings emphasize the host´s regulatory role in shaping microbiome composition and abundance in response to nutritional inputs.

A particularly compelling explanation involves the metabolic flux generated by feeding, which can reshape the physicochemical microenvironment of host tissues. In mammals, nutrient intake activates mTOR signaling and drives glycolysis and lipid biosynthesis, generating host-derived metabolites that fuel microbial growth (192, 193, 194). mTOR complexes, particularly mTORC1, are activated by amino acids and other nutrients, coordinating cellular process with nutrient availability. This nutrient-sensing mechanism extends to the gut microbiota, where host-derived metabolites fuel microbial growth (195). This interplay between gut microbiota and mTOR signaling influences various physiological functions and disorders, including obesity, diabetes, and cancer (196, 197). Complex transcriptomic responses to feeding and starving was revealed with studies on juvenile *Nematostella vectensis* (198, 199). Starvation downregulates genes involved in metabolism, cellular respiration, and immunity (199). Starvation also reduces NF-κB levels and increase susceptibility to bacterial infection (199), suggesting that the immune system enters low-activity state under nutrient limitation. Such immune dampening could reduce immune-mediated clearance of commensal bacteria, further contributing to the reduced microbial load observed in these animals. This pattern aligns with observations in other invertebrates and vertebrates, where nutritional stress suppresses immune gene expression, thereby shifting the balance between tolerance and defense (200, 201). Additionally, in other cnidarians, starvation affects gene expression, immunity, and pathogen susceptibility differently in symbiotic and aposymbiotic cnidarians (202). This pattern has recently been observed in *Nematostella* juveniles as well. Steinmetz and colleagues (198) showed that juvenile polyps undergoing cycles of starvation and refeeding exhibited strong plasticity in both physiological and transcriptomic profiles, including metabolic reactivation upon refeeding and suppression of immune and developmental pathways during starvation. These shifts provide further support for a model in which nutrient availability acts as a central regulator of immune tone and microbial permissiveness throughout development, reinforcing the evolutionary and

functional relevance of starvation-induced immune modulation in this species (198). Importantly, Steinmetz et al. also reported downregulation of key immune components—including pattern recognition receptors, NF-κB signaling elements, and effector molecules—during starvation phases, suggesting that juveniles may suppress their immune system to conserve energy and prevent excessive inflammation in the absence of food. Upon refeeding, a rapid reactivation of immune-related genes was observed, indicating that immune suppression is reversible and tightly coupled to nutrient sensing (198). These findings highlight a developmental window in which nutrient cues shape immune programming, likely with long-term consequences for microbiome establishment and immune homeostasis. It is plausible that in *Nematostella*, starvation constrains the availability of host-derived resources while reducing immune effector activity, as suggested by the concurrent decline in both polyp size and bacterial abundance.

Host selection fosters stress-resilient microbes under starvation

The observed decline in microbial diversity under starvation may reflect not only a loss of dietary-associated bacteria, but also active host selection for core microbial taxa resilient to metabolic stress. Indeed, several studies have identified bacterial lineages that persist or even dominate under nutrient-deprived conditions. For example, members of the Spirochaetaceae family, enriched in starved *Nematostella*, are known to exhibit flexible metabolic strategies, including the ability to survive on host-derived compounds and to endure anaerobic or low-resource environments (203). Notably, Spirochetes lack lipid biosynthesis pathways leading to a disproportionate flux through glycolysis rather than the pentose phosphate pathway (204), which enables Spirochetes to survive in low-resource environments and still maintain their complex life-cycles.

Similarly, *Planctomycetes* and *Chloroflexi*, frequently observed in starved marine invertebrates, have been associated with slow growth rates, resistance to phagocytosis, and capacity for recycling recalcitrant organic matter (205, 206, 207). In corals and sponges, microbial taxa that increase during stress often possess traits linked to dormancy, biofilm formation, or reduced reliance on host-derived nutrients (42, 208). These observations suggest that microbial survival under host starvation may depend on

both intrinsic microbial traits and the host's selective immune filtering. These examples may benefit from metabolic plasticity, slow replication, or evasion of immune detection—traits that render them well-suited for survival in a starved host environment. The persistence of these microbes under immune and nutritional constraint may, in turn, play a stabilizing role for the host.

In cnidarians and other basal metazoans, stress-resistant symbionts have been linked to functions such as nutrient recycling, redox balance, and epithelial maintenance (208, 209, 210, 211, 212, 213). While the functional role of these taxa in *Nematostella* remains to be fully characterized, their retention during stress suggests that the host may rely on a core set of mutualists to buffer against metabolic collapse and preserve epithelial integrity during periods of deprivation. In *Nematostella*, it is likely that starvation imposes a dual pressure—limiting resource availability while enhancing immune scrutiny—that selects for slow-growing, host-adapted taxa capable of persisting in a constrained and immunologically active environment. This is consistent with findings from marine invertebrates such as sponges and mussels, where starvation or caloric restriction leads to microbial simplification favoring slow-growing, tightly associated symbionts. In sponges, like *Aplysina aerophoba*, a large fraction of associated bacteria remains stable during starvation, which indicates a highly integrated relationship (214). Similarly, mussels gut microbiome shows resilience to short-term starvation. However, prolonged starvation in mussels leads to symbiont loss and gill structure changes (215, 216).

From an ecological standpoint, these dynamics may represent an adaptive restructuring of the metaorganism, where the host restricts microbial flux under resource-limited conditions and re-expands symbiotic complexity upon refeeding. In this light, the feeding-starvation axis emerges as a regulatory switch that not only affects host energy balance but also reorganizes the microbiome in ways that optimize host survival under fluctuating environmental conditions. These dynamic underscores a central theme of the first chapter: that *Nematostella* does not passively reflect environmental microbial inputs, but rather engages in active microbial curation depending on its metabolic state. By toggling between permissiveness and selectivity in response to nutrient availability, the host enforces distinct microbial regimes that are ecologically tuned to periods of growth or conservation.

From an evolutionary perspective, such plasticity may represent an ancient and conserved strategy for managing microbial symbionts. Similar nutritional regulation of microbiome composition has been observed in other basal metazoans, such as sponges and ctenophores, where shifts in feeding alter the abundance of functional microbial guilds (217, 218). Even in bilaterians, nutrient-responsive modulation of host-microbe interactions—through changes in host metabolism, immune signaling, or epithelial renewal—points to a deep evolutionary link between feeding behavior and microbiome control (219).

The fact that *Nematostella*, a diploblastic animal lacking a centralized gut or circulatory system, exhibits such sophisticated microbiome restructuring suggests that the origins of immune-metabolic coordination and microbiome filtering extend far deeper in metazoan evolution than previously appreciated.

Discrimination, adaptation, and memory: nematosomes as immune integrators

The second and third chapters explored the immune specificity and adaptive potential of *Nematostella's* nematosomes—phagocytic cell clusters composed of amoebocyte-like cells embedded in extracellular matrix, located within the body cavity and functioning as immune-sensing compartments. These structures, already observed in *Nematostella's* histology but previously underexplored, may represent an ancestral form of organized immune tissue in early-diverging metazoans. Nematosomes are enriched in phagocytic and lysosomal machinery, as demonstrated by proteomic detection of cathepsins and vacuolar ATPases, and by strong uptake of bacterial particles via phagocytosis assays. Isolated nematosomes exhibited phagocytic activity within two hours of bacterial exposure, and their lysosomal compartments were highly acidified in response to microbial contact. This response was sharply diminished in nematosomes from *cJUN* knockout animals, establishing that the AP-1 transcription factor cJUN is critical for both phagocytic activation and downstream degradation. These findings place nematosomes among the earliest examples of multicellular immune effectors with discrimination and functional plasticity (**Figure-D 2**).

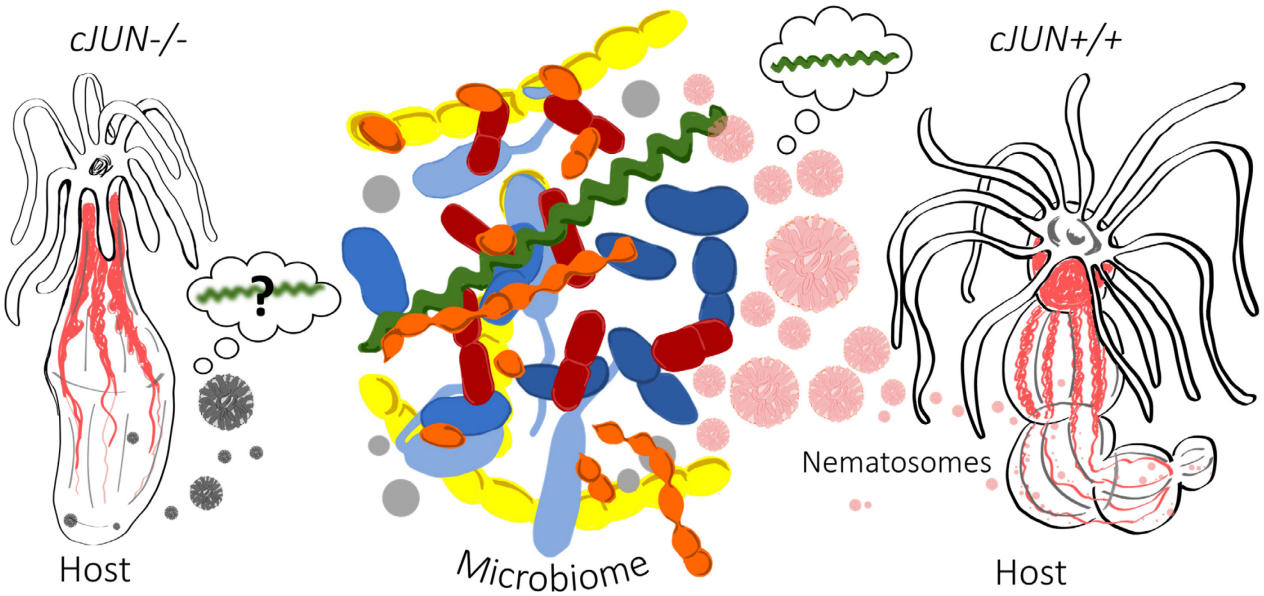

Figure-D 2 cJUN regulates immune-mediated microbial homeostasis in Nematostella vectensis. *Schematic overview depicting the essential role of cJUN in maintaining host immune function and microbiome recognition. In cJUN-deficient animals (cJUN$^{-/-}$; left panel), nematosomes are significantly reduced or impaired, leading to compromised bacterial clearance and resulting in microbial dysbiosis without strain-specific recognition. Conversely, wildtype animals (cJUN$^{+/+}$; right panel) display robust, cJUN-dependent nematosome activation, facilitating effective phagocytosis and immune responses that sustain a balanced and diverse microbial ecosystem. The central panel illustrates the microbiome composition, emphasizing enhanced microbial diversity and stability in wildtype hosts. This schematic underscores the critical role of cJUN as a central coordinator of innate immune mechanisms influencing host-microbiome dynamics.*

Comparable multicellular or tissue-level innate immune structures are seen in other invertebrates. In annelids such as *Eisenia fetida*, coelomocyte aggregates form nodular structures with phagocytic, encapsulating, and memory-like functions (220, 221). Similarly, in tunicates like *Ciona intestinalis*, morula cells function as sentinel-like effectors involved in recognition and storage of microbial products (222, 223). Although structurally diverse, these examples suggest that localized immune compartments capable of coordinated microbial interaction arose multiple times and may reflect a conserved evolutionary strategy predating adaptive immunity.

Strikingly, these cells showed the ability to discriminate between native and non-native microbes. When challenged with a panel of *Vibrio* strains, nematosomes preferentially internalized foreign isolates over those previously associated with the host microbiome,

and this selectivity correlated with reduced colonization success of the non-native strains. This level of immune discrimination is rare in invertebrate models and points to a cell-intrinsic recognition capacity. Although the upstream receptors remain uncharacterized, functional parallels may be drawn with the Mediterranean mussel *Mytilus galloprovincialis*, in which hemocytes discriminate self and non-self bacteria via differential expression of C1q-domain-containing proteins (224, 225), and with *Caenorhabditis elegans*, where the p38 MAPK pathway mediates microbial-specific transcriptional programs (226).

The temporal dynamics of nematosomal responses further revealed that these cells undergo functional adaptation based on microbial history. In a recolonization model, animals pre-exposed to a particular *Vibrio* strain displayed reduced phagocytic activity upon re-exposure to the same strain—without losing responsiveness to novel strains (**Chapter 3**). This strain-specific dampening persisted and was abolished in *cJUN* mutants, suggesting that cJUN is not only a regulator of effector activation, but also a molecular integrator of microbial memory. Such a response resembles immune training and immune tolerance as defined in vertebrates: non-clonal, antigen-unspecific memory mediated by innate immune cells (227) (**Figure-D 3**).

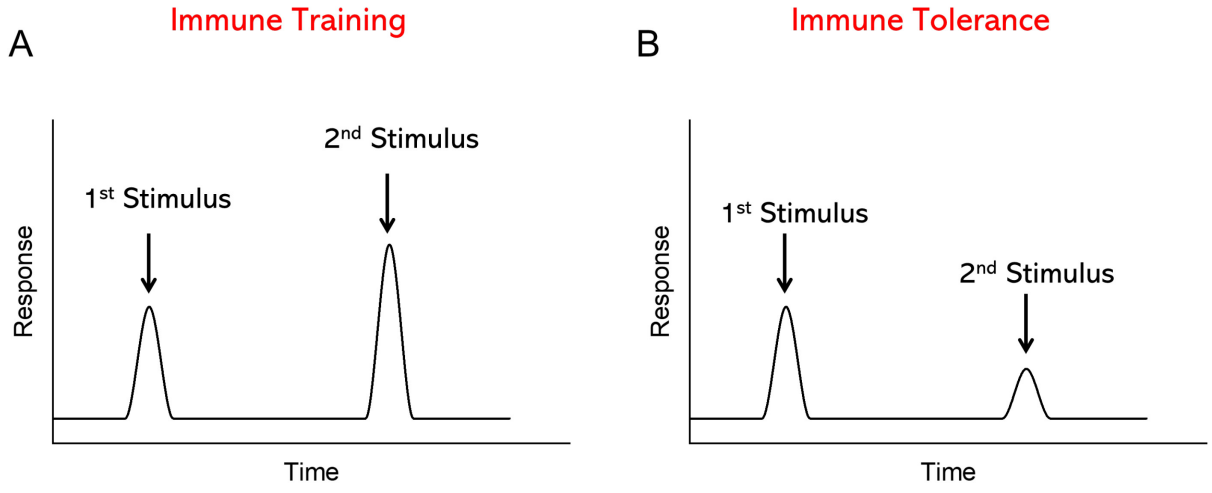

Figure-D 3 Distinct outcomes of innate immune responses upon repeated stimulation. (A) *Immune training is characterized by an enhanced response upon secondary stimulation, indicating a heightened or primed state of the innate immune system following initial exposure.* ***(B)*** *Immune tolerance is characterized by a diminished response upon secondary stimulation, reflecting a suppressed or desensitized state of the immune system. These schematic representations illustrate how prior exposure to a stimulus can differentially modulate subsequent innate immune responses, depending on context, dose, and the nature of the stimulus (adapted from (227, 228))*

Mechanistically, trained immunity in vertebrate macrophages and monocytes involves epigenetic remodeling and metabolic shifts, enabling faster or dampened responses upon re-challenge (229). In *Drosophila melanogaster*, enhanced survival upon secondary bacterial exposure has been linked to sustained upregulation of immune effector genes (230, 231), though chromatin-level regulation remains less defined. In corals it is demonstrated that prior environmental stimulation alters histone accessibility and methylation (232, 233), and similar regulatory plasticity may underpin *Nematostella's* memory-like state. While histone profiling is still lacking for nematosomes, the strain-specific suppression and cJUN dependence suggest a regulatory architecture that supports functional reprogramming.

In vertebrate models, trained immunity is mediated by durable epigenetic marks, including H3K4me3 at promoters and H3K27ac at enhancers, which render innate immune genes more accessible upon secondary stimulation (234, 235). For example, exposure to β-glucan in human monocytes induces persistent histone acetylation at promoters of inflammatory genes such as IL6 and TNF, priming cells for enhanced transcriptional output upon secondary infections (236). In zebrafish, infection-induced training results in lineage-specific reprogramming of hematopoietic progenitors through similar chromatin modifications (237). In the invertebrate snail *Biomphalaria glabrata,* an intermediate host for *Schistosoma mansoni*, DNA methylation changes following parasitic exposure suggest an epigenetic basis for immune priming (238), though causal mechanisms remain to be fully elucidated.

In the context of *Nematostella*, these findings raise the possibility that cJUN functions as both an acute transcriptional activator and a long-term chromatin remodeler. Given its known role in AP-1 complex formation and enhancer selection in other systems, it is plausible that cJUN in nematosomes facilitates chromatin opening at specific immune effector loci during microbial exposure, leaving behind a poised epigenetic state that alters future responsiveness. Although theoretical, this would represent an elegant mechanism through which a single transcription factor mediates both immediate and memory-like functions in a basal animal. Such a model invites future investigations using ATAC-seq (239) or CUT&RUN (240) to identify cJUN-bound regulatory elements and chromatin states before and after microbial challenge.

In macrophages, trained immunity is metabolically underpinned by shifts toward aerobic glycolysis, mitochondrial respiration, and fatty acid synthesis (241, 242, 243). Although *Nematostella* has not been profiled metabolically under bacterial challenge, the elevated lysosomal activity observed post-exposure suggests energetic reallocation consistent with immune stimulation. Similar patterns have been observed in other basal metazoans and invertebrate models. In corals such as *Pocillopora damicornis*, immune stimulation leads to oxidative bursts and enhanced expression of mitochondrial enzymes and redox-associated genes, suggestive of increased metabolic demand (244). Additionally, in the sea urchin *Strongylocentrotus purpuratus*, challenge with microbial components activates coelomocytes and triggers expression of genes linked to mitochondrial energy metabolism and ROS production (245). These examples support the idea that innate immune stimulation often involves bioenergetic shifts across diverse non-vertebrate phyla, potentially enabling functional plasticity and immune memory in the absence of adaptive systems.

These immune adaptations have particular relevance in the dynamic estuarine environments *Nematostella* inhabits. Microbial communities in these habitats fluctuate seasonally and spatially, requiring hosts to continually modulate tolerance and defense. An immune system that can remember microbial identity and selectively dampen responses reduces energetic cost while preserving readiness for novel threats. This dual balance of tolerance and vigilance—encoded not through clonal diversity, but through transcriptional plasticity and effector modulation—reframes how we understand the capabilities of innate immune systems. Immune tolerance is especially critical in barrier tissues like those of *Nematostella*, where constant exposure to environmental microbes requires selective restraint. In other invertebrates such as Hydra, immune tolerance toward commensals has been linked to suppression of antimicrobial peptide (AMP) production and pattern recognition receptor signaling (13, 14). Similarly, oyster hemocytes modulate phagocytic activity and oxidative bursts in a context-dependent manner, enabling a balance between host protection and microbial coexistence (246). Though specific mechanisms remain to be uncovered in *Nematostella*, the observed strain-specific suppression of nematosomal activity suggests that this tolerance is not a passive absence of response, but an actively maintained state, potentially involving negative

regulation via transcription factors like cJUN or immune-modulatory cytokine analogs yet to be identified.

In a speculative but compelling interpretation, one might view the nematosome not only as a local immune effector, but as a primitive analog of hematopoietic immune niches seen in higher animals. While lacking the cellular renewal properties of bone marrow, nematosomes may act as semi-autonomous hubs for microbial sensing, memory encoding, and effector deployment—akin to how vertebrate tissue-resident macrophages maintain local immune tone. Their ability to filter, adapt, and store microbial history in a compartmentalized form hints at a deeply embedded logic of immune modularity in early metazoans. If confirmed, such a function would position nematosomes as not merely phagocytic structures, but as multifunctional immune micro-organs at the dawn of animal evolution.

Together, these findings suggest that nematosomes in *Nematostella* act not only as microbial sentinels, but also as context-aware effectors capable of adaptation and recall. Their cJUN-dependence, microbial discrimination, and functional modulation align them conceptually with trained phagocytes in annelids (247), mollusks (248), and even coral immune cells (249). These parallels underscore a conserved evolutionary logic in which innate cells acquire temporary responsiveness shaped by prior exposure. In this light, trained immunity is not an innovation of vertebrates, but a deeply rooted feature of innate immune systems.

Conclusion

This work has charted the complex mechanisms by which the early-diverging animal *Nematostella vectensis* exerts precise control over microbial colonization—illuminating a multi-layered system that integrates nutrient sensing, immune filtering, and transcriptional regulation. Through specialized structures such as nematosomes, flexible signaling cascades, and context-dependent modulation, *Nematostella* reveals an unexpected sophistication in managing its microbial partners. These findings position early animals not as passive habitats, but as active participants in sculpting their microbial landscapes—balancing defense and tolerance with remarkable nuance.

The discovery that nematosomes not only clear microbes but also exhibit memory-like responsiveness to prior microbial encounters redefines our understanding of innate immunity. This capacity for functional reprogramming blurs the classical boundaries between innate and adaptive immune systems, demonstrating that historical experience can shape future responses even in basal metazoans. Far from being simple effector sacs, nematosomes emerge as evolutionary innovations—central players in maintaining microbial homeostasis and enacting forms of immune memory long thought exclusive to vertebrates.

In the dynamic estuarine habitats where *Nematostella* thrives, the ability to modulate microbial colonization with ecological sensitivity is not only adaptive—it is essential. By reframing the innate immune system as a mediator of coexistence, rather than merely a barrier to infection, this thesis advances a broader view of immunity as a dynamic interface between host and environment. Ultimately, these findings contribute to a deeper evolutionary understanding of how animals, from their earliest origins, evolved the means to negotiate life in a microbial world.

Outlook

Building on the findings of this thesis, several promising avenues emerge to further dissect the mechanisms of immune plasticity and host-microbiome integration in *Nematostella vectensis.* First, single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics could be employed to map the cellular heterogeneity and gene expression dynamics of nematosomes during microbial exposure (250, 251). Such approaches would help define whether subpopulations of cells specialize in memory encoding, effector deployment, or immune regulation.

Epigenomic assays such as ATAC-seq or CUT&RUN targeting cJUN and histone marks (e.g., H3K4me3, H3K27ac) could reveal whether trained immune states are encoded at the level of chromatin architecture. Similar approaches have been transformative in vertebrate models: for instance, ATAC-seq mapping has shown that β-glucan-trained human monocytes develop persistent enhancer activation at key inflammatory loci (235), while CUT&RUN profiling has been adapted for zebrafish, enabling high-resolution chromatin profiling during embryogenesis (252). Combined with CRISPR interference or epigenetic editing tools, such assays could uncover causal links between cJUN binding, enhancer activity, and transcriptional persistence.

Metabolomic profiling of *Nematostella* under feeding, starvation, and bacterial challenge could illuminate the energetic costs and substrates underlying immune activation, especially within nematosomes. Stable isotope tracing or Seahorse metabolic flux analysis in isolated tissues could further test whether immune training involves metabolic reprogramming akin to that observed in vertebrate macrophages (253, 254, 255).

Beyond the molecular, ecological and evolutionary frameworks could be integrated. For example, reciprocal transplant or microbial reconstitution experiments across estuarine gradients could test whether nematosomes adapt to locally dominant microbes through region-specific training. Additionally, time-resolved microbiome sequencing during immune training could clarify how host memory shapes community resilience or restructuring.

Finally, synthetic microbial consortia—engineered to vary in taxonomic similarity or immune evasion capacity—could be deployed to probe the specificity, limits, and costs of

immune training in *Nematostella*. This would help quantify the balance between tolerance and defense in shaping the long-term dynamics of host-microbe symbiosis.

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Abschließend erkläre ich, dass mit dieser Arbeit keine andere Dissertationsverfahren, mit oder ohne Erfolg, eingereicht wurde.

Düsseldorf, den

Nida Hatice Kaya