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Prokaryote treatments and coral nutrition: Feasibility of research and recommendations

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1 Introduction

An unprecedented frequency of mass bleaching events seen in the last decade as a direct result of increasing sea temperatures emphasizes the urgency of actions to protect our coral reef systems. In their 2021 Policy Paper, the International Coral Reef Society defined three pillars of action that are needed to face these threats (Knowlton et al., 2021). The first pillar is a reduction of global climate threats, which are essential and without which the other actions may become irrelevant. The second pillar is improvement of local conditions to reduce the overall or cumulative stress from factors such as changes in land use, pollution, direct exploitation and invasive species. Finally, the third pillar is active restoration to support the survival of reefs while the effects of transitioning to a low carbon economy come into effect.

One approach to active coral restoration is seeding with sexually produced coral, which increases the genetic diversity of deployed stock relative to asexually produced coral fragments (Knowlton et al., 2021; Randall et al., 2020). This can be achieved through larval-based restoration or by coral seeding (McLeod et al., 2022), with each strategy presenting their own advantages and challenges as discussed in detail elsewhere (Banaszak et al., 2023; McLeod et al., 2022; Randall et al., 2020). Coral seeding using spat (single polyps) or juveniles produced in *ex situ* aquaculture facilities present advantages including the opportunity to control the species and genetic diversity of the seeded corals, and to incorporate strategies such as stress hardening, selective breeding, and inoculation with temperature-tolerant symbionts to produce corals that are more resilient to future climate conditions (Randall et al., 2020). Additionally, *ex situ* aquaculture production provides the opportunity to control the availability of appropriate settlement inducers, and to include a protective grow-out phase where the survival, growth and health of coral juveniles can be supported through the control of competitors and the provision of nutrition and probiotic treatments (Banaszak et al., 2023; McLeod et al., 2022; Randall et al., 2020).

Research and development undertaken by the Reef Restoration and Adaptation Program has made significant progress in the automation of *ex situ* spawning and fertilisation of corals by the development of the AutoSpawner system (Severati et al., 2024). These developments have already reduced the time demands and labour costs associated with producing coral larval cultures by up to 113-fold as compared to traditional methods (Severati et al., 2024). However, key bottlenecks remain relating to successful settlement of sexually produced coral larvae and post-settlement survival in the facility and after deployment onto the reef (Banaszak et al., 2023; Randall et al., 2020)).

This report discusses how RRAP-funded projects have progressed towards addressing these bottlenecks through the identification of bacterial inducers to enhance coral settlement, and the development of bacterial probiotics and coral diets to support the survival, growth, and health of coral spat and juveniles. It examines the feasibility of these approaches by discussing the challenges and opportunities for their application to support coral aquaculture at the scale required for effective reef restoration. Finally, the report evaluates critical risks and research gaps and provides recommendations for future work.

2 Microbial inducers for coral larval settlement

2.1 General introduction and underlying principles

Coral larval settlement is a critical step in the coral life cycle during which planktonic larvae attach to the substrate and undergo metamorphosis to develop into juvenile corals. Coral sexual propagation depends on the ability to efficiently cue settlement and the lack of reliable settlement cues remains a major bottleneck in coral aquaculture, particularly for many important and diverse non-acroporid species that are typically less-well studied compared to acroporid species (Banaszak et al., 2023; Bostrom-Einarsson et al., 2019; Randall et al., 2020). Crustose coralline algae (CCA) and microbial biofilms are currently the most well-studied sources of settlement cues. While CCA are effective inducers of larval settlement for many coral species (Abdul Wahab et al., 2023; Ritson-Williams et al., 2014; Whitman et al., 2020), harvesting live CCA from the reef for use in coral aquaculture is not sustainable at scale, and new solutions to enable large-scale aquaculture settlement are required.

Microbial biofilms have been shown to induce larval settlement in a diverse range of coral species (Padayhag et al., 2023; Randall et al., 2024; Webster et al., 2004) and other marine invertebrates (Cavalcanti et al., 2020; Hadfield, 2011). However, due to the high diversity and complexity of biofilm communities, the specific taxa responsible for generating settlement cues remain largely unknown. Only a few inductive bacteria have been identified for limited coral species (Negri et al., 2001; Petersen, Moeller, et al., 2021; Sneed et al., 2014; Tran & Hadfield, 2011), but these examples are able to induce settlement at rates equivalent to or even exceeding that of natural biofilms or CCA (Petersen, Moeller, et al., 2021; Tran & Hadfield, 2011). Since microbes can be readily cultured, they present a promising opportunity for the development of novel settlement-inducing treatments, either through use of inductive microbes themselves or application of microbial-produced settlement cues (Banaszak et al., 2023).

2.2 State of knowledge

2.2.1 Settlement substrates commonly used for the sexual production of corals in reef restoration

Coral larvae reared for reef restoration are generally settled on artificial substrates or devices that have been biologically conditioned in the ocean or land-based aquaria to develop biofilms and CCA communities (e.g., (Chamberland et al., 2015; Guest et al., 2014; Miller et al., 2022). However, there are several drawbacks to this approach: 1) it requires long conditioning times (typically over weeks to months); 2) the resulting biofilm communities can be highly variable yielding unpredictable or non-replicable settlement results, 3) it is not effective for the full range of coral species required to restore biodiverse reefs; and 4) it can result in overgrowth of other benthic organisms that compete with juvenile corals and negatively impact their long-term survival (Banaszak et al., 2023). Some of these drawbacks can be mitigated, for example by co-culture with herbivores to reduce algal overgrowth (Neil et al., 2021) or use of antifouling coatings (Roepke et al., 2022), but the scalability and reliability are more difficult to address. Applying settlement inducing microbes or biochemical cues produced by CCA or biofilms directly to settlement devices has thus been proposed as a solution to overcome these limitations (Banaszak et al., 2023; Morse et al., 1994; Randall et al., 2020). However, while several microbial settlement cues have been partially or fully isolated from both CCA and biofilms (reviewed in Turnlund et al. *submitted*), none have yet been successfully developed into treatments suitable for use in coral aquaculture.

2.2.2 Known microbial inducers of coral settlement

Several strains of bacteria have been shown to induce coral settlement either when grown as biofilms on various settlement substrates (Petersen, Moeller, et al., 2021; Tran & Hadfield, 2011) or when added directly to the seawater (Sharp et al., 2015; Zhang et al., 2024). The majority of these inductive strains belong to the phylum Pseudomonadota, particularly the genus *Pseudoalteromonas*, but they also include Actinobacteria, Bacteroidota, and Bacillota (Petersen, Moeller, et al., 2021; Sharp et al., 2015; Tran &

Hadfield, 2011; Ying Zhang et al., 2021). In most cases the specific cues and mechanisms by which these bacteria induce settlement are unknown. To date, only two fully characterised chemical cues produced by bacteria have been described: tetrabromopyrrole (TBP) and cycloprodigiosin (CYPRO). TBP is a brominated aromatic hydrocarbon first isolated from the CCA-associated *Pseudoalteromonas sp.* PS5 strain (Tebben et al., 2011). While it can induce high rates (38-84 %) of complete settlement (i.e., attachment and metamorphosis) in some coral species (Sneed et al., 2024; Sneed et al., 2014), in others it largely induces metamorphosis without prior attachment to the substrate (Tebben et al., 2015; Tebben et al., 2011) which is ultimately fatal to the larvae. CYPRO is a red alkaloidal pigment isolated from a strain of *Pseudoalteromonas rubra* that induces complete settlement in the brooding coral *Leptastrea purpurea* at rates as high as 90% but has not been tested in other species (Petersen et al., 2023; Petersen, Kellermann, et al., 2021). Both compounds are light sensitive and chemically unstable, which poses challenges for their use in coral aquaculture. Notably, both compounds were isolated from strains of *Pseudoalteromonas*, which are often found at low abundances in natural biofilms communities suggesting these compounds are unlikely to be key drivers of larval settlement under natural conditions (Tebben et al., 2015). Thus, there are expected to be other microbial settlement cues that remain undiscovered.

2.2.3 Desirable properties of microbial inducers for upscaling

Research to date has demonstrated that bacterial inducers can be highly effective at inducing settlement in a range of coral species. However, more fundamental research is needed to identify reliable settlement cues that target the full range of desired coral species and are suitable for production and application at the scale required for reef restoration (Randall et al., 2020). Factors that should be considered when evaluating the potential of a settlement cue for use in large-scale coral aquaculture include effectiveness and range of action, mode and cost of upscaled production, chemical properties, stability and shelf-life, ease of application, and potential health or environmental risks (Table 1). Ideal settlement cues will be effective for a wide-range of corals, low cost and readily produced at scale, stable for transport and long-term storage, and easily applied to desired settlement surface with no off-target health or environmental effects. Cues that meet most of these optimal requirements have the potential to greatly improve the efficiency of coral sexual propagation for reef restorations.

Table 1: Microbial settlement cue properties and factors to consider when evaluating their potential application in large-scale coral aquaculture for reef restoration.

Settlement cue properties	Considerations for upscaling production and application
Bacteria vs bacteria-produced cues	Live bacterial inducers can be identified and used more quickly. While bacteria-produced cues require further work to identify and isolate the specific settlement-inducing compounds, they may be more practical for application in large-scale aquaculture.
Mode of production	The mode of production will impact the ease, time, and cost of producing settlement cues. To meet the demands of large-scale reef restoration, these cues must be amenable to large-scale production, likely in partnership with industry.
Cost and feasibility of upscaling	Can sufficient quantities be produced at a low enough cost to make widespread application in reef restoration feasible, especially considering potential funding limitations and specific cost targets per coral?
Effectiveness and Range of action	Settlement cues should induce high rates of settlement to ensure efficient conversion of sexually propagated larvae to settled spat. Broad-acting cues that induce settlement across multiple coral species would have wide applicability. However, species-specific cues that target endangered or difficult-to-settle species

	may prove beneficial.
Chemical properties	Chemical properties will affect the behaviour of a settlement cue. For example, whether a cue is hydrophilic or lipophilic will determine its solubility in seawater, therefore influencing how it interacts with larvae during settlement.
Stability and shelf-life	Is the cue stable once produced or applied to settlement devices and what is its shelf-life? Cues with a longer shelf-life will be more practical for offsite production, transport, and long-term storage compared to those that must be produced on demand for immediate use.
Impacts on coral health and survival	Does the cue have any long-term impacts on coral health or survival after settlement?
Environmental risks	Does the settlement cue pose potential risks to the health of non-target organisms or the environment? If so, can these risks be mitigated?

2.3 Approach

One challenge in identifying specific microbial inducers from complex biofilm communities is that biofilms are highly diverse, often consisting of >1000 of species of microorganisms (Qian et al., 2022). The conventional approach is to cultivate bacteria from CCA or biofilms and systematically test each isolate for inductive activity in larval settlement assays (e.g., Negri et al. (2001); Tebben et al. (2015); Tran and Hadfield (2011). However, this is time consuming and often yields low success rates. Alternatively, sequencing-based techniques combined with statistical analyses of complex biofilm communities may help identify taxa or cues correlated with coral settlement (reviewed in Turnlund et al. (Submitted)). For instance, Turnlund et al. (2023) used co-occurrence network modularity analysis to show that as many as 20 species of microorganisms in one biofilm were associated with high settlement of *Acropora tenuis* larvae. Similarly, Padayhag et al. (2023) employed a high throughput correlation approach to identify hundreds of microbial taxa correlated with *A. tenuis* settlement. These analyses to identify novel inducers could then guide cultivation efforts to target the specific microorganisms. Hence, in RRAP phase 1, we aimed to leverage this targeted approach by first identifying bacterial inducers, then cultivating these bacteria for validation experiments to test their settlement activity and determine those most suitable for developing treatments to enhance settlement in aquaculture.

2.3.1 Identification of candidate bacterial inducers

To first identify potential bacterial inducers, larval settlement assays were performed with 6 coral species using microbial biofilms conditioned under different treatments (light vs dark) and over different time-periods (1–2 months) to identify high and low settlement biofilms. Biofilm communities were then characterised using 16S rRNA gene amplicon sequencing to determine taxonomic profiles and metagenomic sequencing for functional profiles. Statistical analyses of biofilm communities revealed numerous taxa correlated with high and low coral settlement (O'Brien et al., In review). Additionally, more than 700 bacterial genomes were recovered and putative bacterial functions linked to settlement suggesting possible mechanisms by which biofilm communities may influence coral settlement.

2.3.2 Cultivation of candidate bacterial inducers

In parallel, these candidate inducers were targeted for cultivation from seawater and biofilms using a range of high and low nutrient media, including specialised media to target key groups that do not readily grow on standard media. These extensive cultivation efforts yielded a total 519 isolates. Taxonomic identification by 16S rRNA gene Sanger sequencing indicated these included 366 unique bacterial isolates from 42 families and 7 phyla. Importantly, these isolates included 201 strains that had >97% sequence similarity to taxa we previously identified to be correlated with high settlement or that were previously shown to

induce coral settlement in the literature (Petersen, Moeller, et al., 2021; Tebben et al., 2011; Tran & Hadfield, 2011).

2.3.3 Experimental screening of candidate bacterial inducers

The 201 identified candidate inducers were experimentally validated in settlement assays with nine coral species to evaluate their potential to induce settlement *in vivo* (Figure 1). In total, ~25% (~50 isolates) were confirmed to induce a larval settlement response in one or more coral species. Some isolates induced settlement in as many as six different coral species (including acroporid and non-acroporid species) suggesting they may have broad action. These inducing bacteria were phylogenetically diverse, representing species from 20 families and 3 phyla, and include many taxa that have not previously been reported to be associated with coral settlement in the literature. This highlights the success of this approach in identifying novel microbial inducers. Optimisation of isolates to maximise settlement is ongoing, and the next steps will include genomic and chemical characterisation to identify the specific mechanisms responsible for settlement inducing activity. This will allow us to select the most promising settlement cues for development into settlement treatments for coral aquaculture.

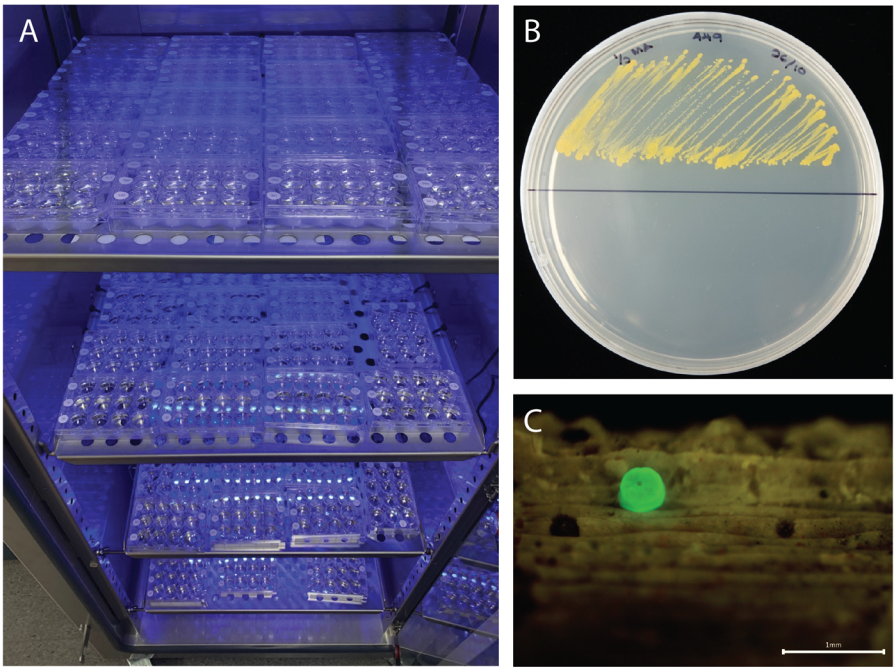

Figure 1*: Experimental screening of candidate bacterial settlement inducers: A) larval settlement assays conducted in well-plates, B) example of a bacterial isolate growing on an agar plate, C) a newly settled coral on a biofilm covered settlement tab, visualised under blue light.*

2.4 Challenges and opportunities for use in large-scale coral aquaculture:

2.4.1 Upscaled production of bacterial inducers

One advantage of bacterial settlement inducers is that bacteria can be readily cultured at scale to meet the demands of reef restoration. Commercial production of bacteria is routinely conducted in industrial scale fermenters in volumes >10,000 L for the production food, cosmetics, chemicals, and fuels (Crater & Lievense, 2018; Lee et al., 2023) and could be adapted to produce marine bacteria (see section 4.4.1 for further considerations in adapting existing fermentation systems for the production of marine bacteria). Bacterial fermentation can be optimised to maximise cell density or tailored to maximise production of a desired metabolite to further improve production efficiencies (Choi et al., 2019; Lee et al., 2023).

There are also multiple potential modes of production of bacterial cues: 1) cues can be extracted and isolated directly from bacterial biomass (e.g., CYPRO (Petersen et al., 2023; Petersen, Kellermann, et al., 2021); 2) some cues can be chemically synthesized without the need for live bacteria cultures (e.g., TBP (Sneed et al., 2024; Tebben et al., 2011); 3) certain cues may even be commercially available (e.g., the GLW-amide peptide Hym-248 (Erwin & Szmant, 2010; Randall et al., 2024), and finally 4) in the case of cues produced by difficult to grow bacteria that cannot otherwise be synthesized it may be possible to use recombinant protein or secondary metabolite production to generate the desired cue using well-established bacterial expression systems that can support high yield production (Ahmed et al., 2020; Lee & Kim, 2015). The most efficient mode of production will need to be evaluated for each specific cue.

2.4.2 Use of bacteria vs bacterially produced cues for settlement treatments

Although either the inducing bacteria themselves or bacteria-produced cues may be used for settlement treatments, bacteria-produced compounds may be more suitable for large-scale coral aquaculture. The use of live bacteria poses logistic challenges for treatment production, transport, and storage as bacterial cultures cannot readily be stored for extended periods of time and therefore may need to be produced on-demand for immediate application. This may be difficult to coordinate with the limited window of availability of competent larvae (Randall et al., 2024). Alternatively, methods may be found to stabilise bacteria for subsequent application (refer to section 3.4.1) or to preserve biofilms once applied to settlement devices to extend treatment shelf-life and improve feasibility of use in an aquaculture setting.

The primary challenge with bacterial-produced cues is that once an inductive strain is identified, further research is required to identify the specific cue responsible for inducing settlement. To date isolating settlement cues has proved challenging (Morse et al., 1994) and may be impossible if interaction with live bacteria is required (Ericson et al., 2019). Biochemical cues are typically identified through bioassay-guided fractionation where crude extracts are made from bacterial biomass using ethanol or other solvents and then fractionated for use in settlement assays to identify the active fraction which can then be chemically characterised to identify the specific cue e.g., (Petersen, Kellermann, et al., 2021; Tebben et al., 2011). This process is laborious and dependent on iterative screening, which proceeds slowly when the larvae of many coral species are only available during annual mass spawning events. High-throughput chemical analyses that can rapidly screen thousands of samples, such as those used in drug discovery, may offer opportunities to improve the efficiency of this process (Dueñas et al., 2023). Nevertheless, the potential benefits of isolating bacterial-produced cues are expected to justify the additional time and effort.

2.4.3 Optimisation of bacterial inducers for settlement treatments

Treatments will need to be optimised to maximise settlement rates and determine the best methods for application onto settlement devices. Quantities of bacterial cultures or inducing compounds will need to be carefully evaluated as studies indicate that concentrations below a threshold fail to yield substantial settlement while concentrations that are too high can be fatal (Petersen, Kellermann, et al., 2021; Sneed et al., 2024; Tran & Hadfield, 2011). For example, Tran & Hadfield (2011) inoculated settlement tiles with different cell concentrations of the inductive strain *Pseudoalteromonas luteoviolacea* and found biofilms formed using higher cell densities (up to 10^8 cells per ml) resulted in an increase in settlement rates from 14 to 68%, but concentrations above this were lethal. Some cues may also be more effective in combination than when applied individually. For instance, multi-species biofilms can induce higher rates of settlement than the single-species biofilms (Petersen, Moeller, et al., 2021). Experimental conditions can further influence the efficacy of settlement cues as demonstrated by the chemical cue CYPRO, which is light sensitive and requires specific lighting parameters to be effective (Petersen, Kellermann, et al., 2021). Coral responses to settlement cues are often species-specific and thus treatments may need to be tailored for each species (Sneed et al., 2024). Optimisation will also be required for each step up in aquaculture production scale as settlement cues are typically initially tested in settlement assays performed in small petri dishes or well-plates and conditions may need to be adjusted for mass settlement in aquaria.

2.5 Associated risks

Despite their potential application in reef restoration, limited research has been conducted into the potential safety risks of microbial settlement treatments. Comprehensive risk assessments will need to be conducted to ensure their safe use in aquaculture facilities and minimize environmental risks of deploying settled corals onto the reef. Live bacteria pose particular risk for the introduction of pathogens and could have unintended consequences on coral health, especially if spat are subsequently colonised by the inducing bacteria (refer to section 3.5 for further discussion on the risks of microbiome manipulation and environmental stewardship). To mitigate these risks, inducing bacteria will need to be rigorously screened (e.g., genomically and experimentally) to eliminate pathogens and ensure they do not pose any off-target or environmental risks (Peixoto et al., 2022; Sweet et al., 2017).

The use of bacteria-produced cues would eliminate some of the risks of using live bacteria but would still require ecotoxicology screening to determine long-term effects on coral health and survival. Most studies only monitor coral larvae for 24–48h after the provision of settlement cues to determine the proportion that settle (e.g., (Petersen, Moeller, et al., 2021; Sneed et al., 2024; Tran & Hadfield, 2011) and few continue to follow their development post-metamorphosis. However, a recent study found that coral spat induced to settle by a strain of *Metabacillus indicus* had higher survival rates 15 days post-settlement compared to controls settled without bacteria (Zhang et al., 2024). Future studies should incorporate more long-term monitoring to ensure coral spat and juveniles continue to develop normally.

2.6 Recommendations

Microbial settlement treatments have great potential to improve the efficiency and success of large-scale sexual propagation of corals for reef restoration. During RRAP phase 1, we have made significant progress in identifying bacterial inducers for a range of coral species. We recommend continuing the development of these inducers into treatments suitable for use in reef restoration. Here we provide next steps and recommendations for research priorities:

- **Prioritise bacteria-produced cues over live bacteria.** Live bacteria pose greater risks and logistical challenges for implementation at scale. Efforts should therefore focus on bacteria-produced cues or methods to preserve bacteria to ensure treatments are practical for use in large-scale coral aquaculture.

- **Identify and isolate specific bacteria-produced cues.** Further research will be required to identify and isolate the specific bacteria-produced cues responsible for inducing coral settlement. The potential benefits of this research are expected to outweigh the additional efforts and cost.

- **Consider feasibility of upscaled production and implementation.** Selection of settlement cues for treatment development should consider the factors outlined in this report to ensure development of a product with potential for upscaling. Feasibility for upscaled treatment production and implementation will depend on securing a suitable industry partner and require a full cost-benefit analysis.

- **Test on a wider range of coral species.** Treatments should be tested and optimised to target as wide a range of species as possible to support restoration of biodiverse reefs. However, specific treatments that target endangered or difficult to settle species may also prove valuable for restoration efforts.

- **Conduct larger scale settlement experiments.** The best methods for applying treatments to desired settlement devices will need to be established and settlement experiments should be scaled up to test efficacy of treatments during mass settlement in aquaculture

- **Conduct longer-term experiments for risk assessments.** Treatments will need to be evaluated to ensure they are safe to use in an aquaculture settling. This should include rigorous genetic and experimental screening as well as longer-term studies to assess health and survival of coral spat and juveniles.

- **Fundamental research to understand mechanisms of bacteria-induced coral settlement.** Studies should investigate bacterial and host gene expression and metabolites to understand the molecular mechanisms underpinning how bacteria influence coral settlement. This fundamental knowledge would inform treatment development and risk assessment.

3 Probiotics for aquaculture production of coral

3.1 General Introduction and underlying principles

The survival, health, and resilience of coral spat and juveniles are critical factors for successful aquaculture-based reef restoration. Bacterial probiotics are commonly used in large-scale production facilities for other marine organisms, such as fish and prawns, to enhance water quality, growth, feed conversion rates, and disease resilience in produced stock (Mohapatra et al., 2013). The development of probiotics for coral aquaculture is however in its infancy (Thatcher et al., 2022). Proof-of-concept studies have shown that supplying bacterial probiotics to captive corals can alter their microbiome and increase bleaching tolerance in adult corals of some species (Damjanovic et al., 2019; Rosado et al., 2019; Santoro et al., 2021). Much less is known about the possible benefits of applying probiotics during coral early-life development, when the coral microbiome is more flexible and diverse (Epstein et al., 2019; Littman et al., 2009; Zhou et al., 2017). Inoculation of early coral life stages in an aquaculture facility offers an opportunity to shape the coral microbiome and introduce bacterial species with beneficial traits, with logistical advantages over probiotic field applications. To assess the feasibility of using probiotics to introduce desired traits into coral early life stages, ultimately enhancing coral survival in aquaculture production facilities and after deployment, several knowledge gaps must be addressed. These knowledge gaps include, but are not limited to, the selection of probiotic candidates, their delivery and retention in coral spat and juveniles, the nature of their interactions with other members of the holobiont, and their effects on coral survival, growth, health and resilience.

3.2 State of knowledge

3.2.1 Corals are associated with diverse microbial communities

Corals harbour a broad diversity of microorganisms, including microeukaryotes, bacteria, archaea, fungi, and viruses (Bourne et al., 2016). The biological unit comprising the animal host and its associated microorganisms (i.e., the microbiome) is termed the coral holobiont (Rohwer et al., 2002) . While the critical role of the microalgal symbionts (family Symbiodiniaceae) within the coral holobiont is well-characterized (Pogoreutz et al., 2020), insights into the relationship between the host and associated bacteria have only emerged in the past few decades (Rohwer et al., 2002). It has become evident that bacterial symbionts play essential roles in nutrient cycling, stress resilience, and pathogen defence, thereby contributing significantly to coral fitness and survival (reviewed in (Bourne et al., 2016; Peixoto et al., 2017; Voolstra et al., 2024).

3.2.2 Flexibility of coral-associated bacterial communities

The relationship between the coral host and associated bacteria is highly dynamic, as bacterial communities are sensitive to the physiological status of the host and environmental disturbances (Glasl et al., 2017; Hernandez-Agreda et al., 2016; Pratte et al., 2018; Sweet & Bulling, 2017; Ziegler et al., 2017). While the breakdown of this relationship, referred to as dysbiosis, can lead to a decline in coral health (Frias-Lopez et al., 2002; Gil-Agudelo et al., 2006; Jones et al., 2004; Sato et al., 2013; Zaneveld et al., 2016), changes in the composition of the bacterial microbiome also allow the holobiont to acquire new traits (Voolstra & Ziegler, 2020; Webster & Reusch, 2017). As the Coral Probiotic Hypothesis (CPH) posits, such flexibility in coral-bacterial associations may enable the holobiont to adapt to environmental perturbation more rapidly than through natural selection on the host genome alone (Reshef et al., 2006).

3.2.3 Probiotics as a tool to enhance coral health and resilience

Leveraging on the flexibility of coral microbiomes and the effective use of probiotics in other wildlife, application of coral probiotics has been proposed as an intervention strategy to mitigate the negative impacts of climate change and other anthropogenic stressors on coral reefs (Peixoto et al., 2017; van

Oppen et al., 2015). The use of probiotics in adult corals to mitigate the effects of heat stress (Li et al., 2023; Rosado et al., 2019; Santoro et al., 2021), pathogen challenge (Rosado et al., 2019; Ushijima et al., 2023) and oil pollution (Santos et al., 2015; Silva et al., 2021) has shown promising results. For example, Rosado et al. (2019) selected a consortium of native coral bacteria based on their ability to perform nutrient cycling, mitigate toxic compounds, and exhibit antagonistic activity against pathogens. Application of this consortium to corals challenged with the pathogen *Vibrio coralliilyticus* partially mitigated coral bleaching (Rosado et al., 2019). Santoro et al. 2021 reported reduced bleaching and mortality in corals exposed to heat stress following inoculation with a bacterial consortium screened for similar beneficial traits as in Rosado et al (2018). In both studies, applying probiotic consortia restructured the coral microbiomes.

3.2.4 Probiotics for coral early life stages

Coral probiotics research has largely centred around adult corals, yet there is emerging evidence that early life stages – larvae, spat and juveniles – have more dynamic and diverse microbiomes than adult corals (Bernasconi et al., 2019; Chan et al., 2019; Damjanovic et al., 2020; Damjanovic et al., 2019; Epstein et al., 2019; Lema et al., 2014; Littman et al., 2009; Zhou et al., 2017). Studies have also demonstrated that corals in their early life stages readily uptake bacteria from the environment (Bernasconi et al., 2019; Ceh et al., 2013; Damjanovic et al., 2020; Damjanovic et al., 2019). For example, Damjanovic et al. (2019) showed that inoculating coral spat and juveniles with a non-native bacterial consortium markedly altered their microbiomes, with DNA sequences of the inoculated strains detected in varying proportions 36 hours after the final inoculation.

Given the dynamic nature of the microbiomes in coral early life stages and their ability to readily uptake bacteria from their environment, coral larvae and spat may be more receptive to microbiome manipulations. This microbiome malleability may present an ideal opportunity to produce resistant and resilient corals for reef restoration through probiotic administration. However, fundamental questions remain on the selection of bacterial species, the physiological state of the bacterial inoculum, and the frequency and method of inoculation. In RRAP v1, we aimed to address key knowledge gaps critical for developing effective coral probiotics and testing the feasibility of applying them at scale in an aquaculture setting.

3.3 Approach

3.3.1 Generation and selection of probiotic candidates

The development of probiotic treatments for aquaculture production of coral for use in reef restoration relies on access to bacteria isolated from local corals. An extensive collection of bacteria isolated from both acroporid and non-acroporid corals from the GBR was therefore produced in this project (**Figure 2**). The taxonomic diversity of recovered isolates was enhanced by using multiple isolation approaches, including general heterotrophic culture media with high and low nutrient contents, as well as targeted isolation strategies to recover bacteria that produce antibacterial compounds or spores – traits commonly found in effective aquaculture probiotics (Ringø, 2020). This new collection included over 900 bacterial isolates recovered from the coral species *Acropora millepora*, *Acropora kenti* (formerly *A. tenuis*), *Acropora hyacinthus*, *Platygyra daedalea*, *Goniastrea retiformis* and *Porites lobata*, complementing our existing collection of ca. 150 bacterial isolates derived from GBR corals. While all existing and most new isolates were recovered from adult corals, about 250 were obtained from gamete bundles and spawning water of the coral species *A. millepora*, *A. kenti*, and *P. daedalea*. Isolates in the new collection were tentatively identified by 16S rRNA gene sequencing and represent bacterial taxa from 4 phyla, 15 orders, and 27 families. The combined collection includes taxa previously used as aquaculture probiotics (Akhter et al., 2015; Aly, 2009) or tested in coral probiotic studies (Rosado et al., 2019; Santoro et al., 2021; Y. Zhang et al., 2021), as well as other taxa known to include members that carry genes for presumed beneficial traits

such as dimethylsulfoniopropionate (DMSP) metabolism, nitrogen cycling, and bacterial photosynthesis (Peixoto et al., 2021; Sweet et al., 2021).

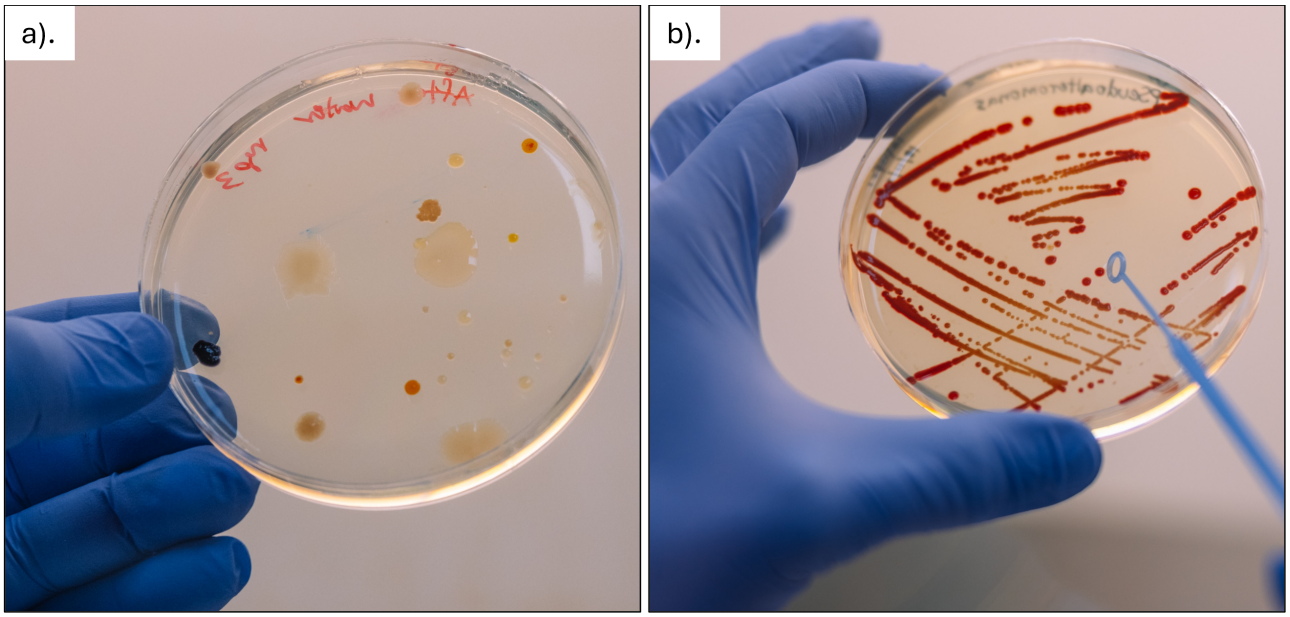

***Figure 2**: Isolation of bacteria from corals. a). Marine agar plate with diverse bacterial colonies from spreading coral material. b). Marine agar plate with streaked colonies of a pure bacterial isolate. Photo credit: SkyReefPhotos.*

The selection of probiotic candidates from the combined culture collection was guided by the taxonomic identity of isolates, with a preference for isolates of the orders Alteromonadales, Flavobacteriales, Hyphomicrobiales, Oceanospirillales, and Rhodobacterales. These orders include bacteria that form close associations with corals (Huggett & Apprill, 2019), and except for Flavobacteriales, bacteria that have been successfully used as probiotics in aquaculture (Akhter et al., 2015; Aly, 2009). To further narrow down candidates for *in vivo* screening, we screened and found: (i) antibacterial activity against the coral pathogen *Vibrio coralliilyticus* in 72 isolates, and (ii) production of one or more enzymes that may enhance the digestion of complex compounds acquired from heterotrophic feeding, including proteins (amylase, caseinase, gelatinase), polysaccharides (chitinase), and lipids (lipase, phospholipase) in 36 isolates. Based on this information, shortlisted isolates were selected for *in vivo* screening and full genome sequencing. The forthcoming genome analysis will provide refined identification of the isolates and enable detailed examination of the putative metabolic pathways associated with the strains (e.g., production of antibacterial compounds, DMSP metabolism, nitrogen metabolism, and bacterial photosynthesis). Additionally, the presence of genes relevant for environmental risk assessments (e.g., toxin genes, antibiotic resistance genes, and evidence of horizontal gene transfer) will be evaluated.

3.3.2 Development of probiotic treatments for coral early life stages

The benefits of using multispecies probiotics rather than individual strains have been repeatedly demonstrated (Goulden et al., 2012; Timmerman et al., 2004), and early work on coral probiotic therapies has utilized probiotic consortia (Rosado et al., 2019; Santoro et al., 2021; Y. Zhang et al., 2021) or transplants (Doering et al., 2021). However, the design of an effective probiotic consortium requires understanding of how individual members associate and interact with the host and other members of the holobiont. For example, it is crucial to avoid including strains that are detrimental to other holobiont members, or indeed to other bacteria in the probiotic consortium. Once such a strain is included in a consortium, it can be challenging to detect and resolve negative interactions.

We therefore performed an initial *in vivo* screening, where individual probiotic candidates were delivered to newly settled coral spat containing photosymbionts (Symbiodinaceaea), in a highly controlled setup using 6-well plates (Figure 3 a) (Thatcher et al., in prep, in prep.). After four repeated bacterial inoculations, the microbiome and coral responses were assessed one and five days after the final inoculation. This experiment was conducted for two coral species (*A. kenti* and *P. daedalea*), testing eight species-specific probiotic candidates, two negative controls, and one placebo control for each coral species. Microbial restructuring was observed in most probiotic treatments, and in four cases the inoculated candidate remained prevalent in the coral microbiome even on the fifth day after inoculation. No significant host recruit phenotype changes were observed over the short duration of this study (17 days), except for one candidate strain that unexpectedly induced tissue loss and mortalities in *A. kenti* juveniles resulting in its elimination as a probiotic candidate. Based on results from this experiment and cross-strain inhibition tests, four probiotic candidates were selected for inclusion in a consortium for subsequent experiments with *A. kenti*.

Identifying effective delivery strategies for coral probiotics is essential for maximizing their successful uptake in the coral holobiont and ensuring feasibility of large-scale application of coral probiotics. We evaluated different approaches to delivering a probiotic consortium to newly settled spat of *A. kenti* held in flow-through systems by assessing both the delivery regime (two or six inoculations) and route (via water or an encapsulated coral diet) (Figure 3 b). The microbiome and host responses were assessed at multiple time points to determine if maintenance additions influenced microbiome dynamics. Then, after the last probiotic application, all treatments and controls were exposed to a short-term heat stress and a recovery period, to assess if the treatments mediated changes in microbiome stability or holobiont resilience to stress. The samples from this experiment have been processed, and data analysis is ongoing.

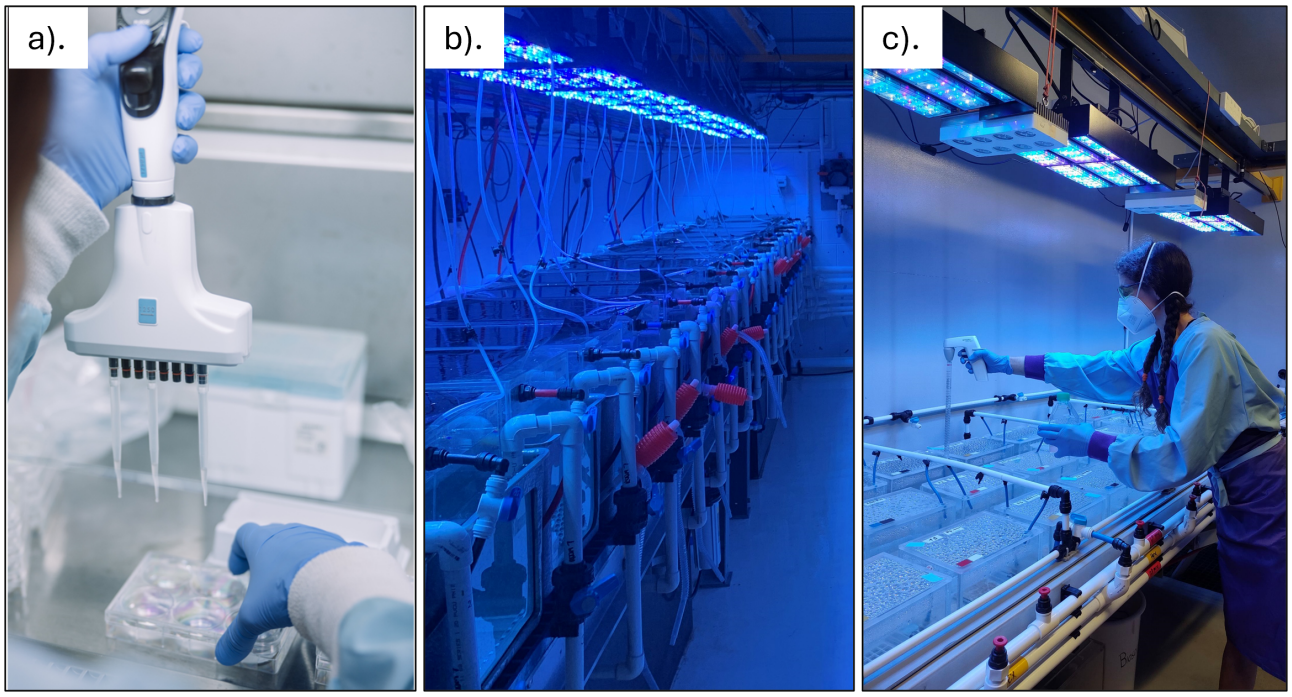

***Figure 3**: Experimental setup for development of probiotic treatments for coral early life stages. a). Six-well plate setup where coral spat settled directly in the plate were inoculated with individual probiotic candidates. Photo credit: SkyReefPhotos. b). 50L flow-through system where coral spat settled on ceramic plugs were inoculated with a probiotic consortium and exposed to a short-term temperature stress. Photo credit: Callaway Thatcher. c). 3L flow-through system where coral spat settled on ceramic tiles were inoculated with individual probiotic candidates and challenged with a putative pathogen. Photo credit: Deepa Varkey.*

In another experiment, we assessed whether select probiotic candidates could enhance the resilience of *A. kenti* spat to pathogen challenge under elevated temperature (**Figure 3** c). The putative pathogen used in the challenge was a *Ruegeria* strain that induced tissue loss and mortality in *A. kenti* spat in the initial 6-well plate experiment described above (Thatcher et al., in prep.). Probiotic candidates were used individually in this experiment to better resolve the complex interactions between the host, the probiotic candidates, and the pathogen. The microbiome and host responses were assessed at multiple time points, including after repeated pathogen challenges combined with heat stress. The last samples from this experiment are currently under analysis.

3.4 Challenges and opportunities for use in large-scale aquaculture

3.4.1 Upscaled production of bacterial cultures

The application of bacterial probiotics and settlement inducers at scale requires large quantities of microbial cultures, necessitating significant specialized infrastructure and expertise for production (see also section 2.4.1). Commercial suppliers of probiotics produce and deliver probiotics in the form of powders, liquids, or slow-release devices or formulations. Upscaled production may influence not only yield, cost, and stability of the final product, but also the physiology and metabolic output of the produced cells (Bianchi et al., 2020; Crater & Lievense, 2018). Therefore, the upscaling process and the properties of the end-product must be carefully assessed and monitored to ensure viability and retention of relevant traits.

Moreover, it is important to consider early the willingness and ability of a potential partner to produce marine bacteria in their fermentation facilities. Most marine bacteria require culture media with elevated salt contents, posing a challenge if fermentation vessels are made of metal or contain metal components that are prone to corrosion. While some marine bacteria can grow in media with lower salt contents, albeit at reduced growth rates, the retention of their relevant functions under these conditions would need to be demonstrated. It may therefore be necessary to optimize culture conditions regarding salt content and consider using fermentation vessels made of plastic. When selecting a potential partner for upscaled production of bacterial cultures, it is necessary to consider factors that may influence the permitting process for coral deployments. These factors include possible existing accreditations of the production facilities by relevant regulators and whether the production facility is located within the same jurisdiction where the probiotics will be deployed.

3.4.2 Development of single inoculation strategies

The probiotic application strategies tested in our experiments involved two to six repeated inoculations of newly settled spat and juveniles, delivered after their uptake of the photosymbiont. This strategy was resource and labour intensive, but the approach was selected to ensure that probiotics were available for horizontal uptake during the time window when the coral microbiome is established (section 3.2.4), and to reduce the risk of interfering with the establishment of photosymbiosis. Preliminary results suggest that some probiotic candidates formed more stable associations than others, and a single inoculation may be sufficient for some candidates. A single inoculation would greatly facilitate large-scale applications by reducing the culture volumes and labour needed. This approach would require validation however, and the optimal time window for the inoculation would need to be determined. Alternative single inoculation approaches delivering probiotic bacteria to coral larval cultures or in combination with photosymbionts could also be explored.

3.4.3 Opportunities for combined inoculation with Symbiodinaceae

Delivery of probiotics to larval cultures would reduce the required culture volume per coral, however this gain would be partially offset if settlement success is low. The establishment of stable bacterial associations may also be less likely in coral larvae due to the subsequent winnowing of the coral microbiome (section 3.2.4), however this process may vary with reproduction strategy (brooders versus broadcast spawners) and across coral species (Bernasconi et al., 2019). A combined delivery of photosymbionts and bacterial

probiotics could also potentially reduce the resources needed overall. It has been shown that bacterial inoculations of the coral photosymbiont *Breviolum minutum* may enhance its thermal tolerance (Heric et al., 2023), however to the best of our knowledge, no studies have yet explored a combined inoculation of corals. Future experiments would need to investigate whether bacterial probiotics influence the stability of the photosymbiosis and associated nutrient exchanges, and whether such a combined inoculation could further enhance coral resilience to heat-stress.

3.4.4 Alternative delivery strategies

The simplest strategy to deliver probiotics to coral is by direct delivery of live cells into the water column, a method that our experiments and others (Silva et al., 2021; Y. Zhang et al., 2021) have shown to be effective. However, this approach requires large culture volumes to achieve typical cell densities used in laboratory experiments (ca 1^6 cells per ml). Typically, 1 ml of culture can inoculate 1 L of seawater, translating to hundreds of liters of bacterial culture at any one time at scaled operations. The development of new tank systems with reduced water volumes per production unit, including for horizontal settlement and for high-density vertical holding of settlement tiles, could make direct inoculation into the water column feasible at large scales. This is contingent upon a commercial supplier producing a stabilized culture that is available as an off-the-shelf product for direct addition to tanks (preferred), or after a short activation step. Delivering probiotics via carriers such as coral feeds is another option, either through enrichment of live feeds (Assis et al., 2020) or adherence to or incorporation into formulated feeds (section 4.4.3). The latter strategy would necessitate additional research to ensure efficient adsorption to diet surfaces, or the use of specialized instruments and/or disinfection protocols to incorporate probiotics during feed production. It is important to note here that direct application onto individual corals, which has been employed in some experiments (Rosado et al., 2019; Santoro et al., 2021) and in the field (Delgadillo-Ordoñez et al., 2024), would be unfeasible at scale without technological advancements to automate the process.

3.5 Associated risks

While microbiome-based interventions hold clear promise for coral conservation initiatives, their use in corals may have unintended consequences that cannot be fully predicted or controlled (Sweet et al., 2017). Changes in microbial photosymbionts can introduce trade-offs in the targeted coral species. For instance, corals that associate with more heat-tolerant algal symbionts, such as *Durusdinium*, gained thermal resilience but suffered reduced growth rates (Jones & Berkelmans, 2010). Whether similar unintended trade-offs occur with changes in coral-associated bacteria remain unknown.

A general risk associated with introducing aquaculture-propagated organisms to the reef is the potential inadvertent release of disease agents into the environment, and this risk applies also to the intentional introduction of beneficial coral microbes if they, or other pathogens or parasites, can spread to or manipulate the microbiome of other native reef organisms or environments (Sweet et al., 2017). For other marine aquaculture species, the emergence of diseases has often accompanied increased production scales and animal densities (Murray & Peeler, 2005), hence this risk is of increasing relevance as coral production is scaled up. We note however that the use of probiotics represents a prophylactic microbial management technique that could potentially reduce the risk of diseases in coral aquaculture facilities (Thatcher et al., 2022).

In the recently proposed science-based framework for stewardship of environmental microbiomes, Peixoto et al (2022) highlights risk assessment as the first step towards implementing microbial interventions for coral restoration. Careful selection of native coral bacteria and exclusion of potential pathogens, such as in our approach, is a key recommendation of the framework as it aligns with the aim of preserving the native reef microbiome (National Academies of Sciences & Medicine, 2019) and poses relatively minimal environmental risk (Peixoto et al., 2022). Nonetheless, whether the selected bacteria could have non-target effects should be rigorously evaluated using a cautious approach including laboratory/aquarium

experiments under controlled conditions, followed by small-scale field trials on reefs with limited connectivity to surrounding systems (van Oppen et al., 2017). Employing such an approach, Delgadillo-Ordonez et al. (2024) reported the successful in situ inoculation of putative probiotic bacteria on healthy coral colonies in the Red Sea. Encouragingly, the coral colonies remained healthy despite a reshaping of their microbiome, and there was no measurable off-target effect on the surrounding seawater and sediment microbiomes (Delgadillo-Ordoñez et al., 2024). While it is critical to assess the risks of microbiome-based intervention strategies, their benefits to coral restoration and the risk of inaction must also be weighed when considering their implementation (Peixoto et al., 2022).

3.6 Recommendations

Probiotics delivered to coral spat and juveniles in aquaculture facilities were demonstrated to modulate, and in some cases were incorporated into, the coral microbiome. Probiotics application in *ex situ* aquaculture can therefore introduce beneficial bacterial traits into the coral holobiont and we recommend continuing the development of these treatments as initiated in RRAP phase 1. Specifically, we recommend that future work include the following activities:

- **Develop single inoculation protocols.** A single inoculation would reduce the resources required for longer-term experiments and reduce logistical challenges for probiotic use in upscaled coral aquaculture. Efforts should focus on identifying the time window during coral ontogeny that is suitable for a single inoculation.

- **Test probiotic strains with a broader range of coral species.** If the same strains can be used for multiple coral species, this would reduce logistical challenges for probiotics use in upscaled coral aquaculture to support restoration of biodiverse reefs.

- **Validate host benefits in longer term experiments.** Benefits to coral survival, growth, health and resilience should be validated in longer term experiments, preferably including deployments with assessment of survival after a natural heat wave or after a longer timeframe (e.g. one year).

- **Investigate further the potential to upscale production of probiotic candidates.** The activity of probiotic candidates after upscaled production would need to be confirmed. Assessments of the costs and logistics of production would be contingent on production volume and securing a suitable industry partner for manufacture and distribution.

- **Conduct fundamental research to understand probiotic mechanisms after inoculation.** Studies should include analyses of bacterial and host gene expression and metabolites, and other advanced methods to assess the localisation of specific probiotics and their metabolic activities. This fundamental knowledge would inform treatment development and risk assessment.

- **Prioritise experiments required to inform risk assessments and the permitting process.** This includes tracking the possible transfer of probiotic strains to non-target organisms, longer-term studies of host performance, and fundamental understanding of probiotic mechanisms (as above).

4 Coral nutrition

4.1 General introduction and underlying principles

The nutritional requirements and heterotrophic feeding behaviours of corals in aquaculture are poorly understood (Banaszak et al., 2023; Conlan et al., 2018; Conlan et al., 2019; Teece et al., 2011), in particular for early life stages (Conlan et al., 2017; Rodd et al., 2022). Developing species-specific heterotrophic feeding regimes for captive corals has the potential to improve survival, growth, and health in the facility and following deployment (Banaszak et al., 2023; Conlan et al., 2017; Conlan et al., 2019; Osinga et al., 2012; Saper et al., 2023). *Artemia* nauplii and microalgae are established food sources for captive corals (Barton et al., 2017; Osinga et al., 2012), but their impact on coral nutritional status is limited and varies between coral species (Conlan et al., 2018; Conlan et al., 2019). Furthermore, published results on the potential benefits of rotifers as feed for captive corals are sparse and available results are variable (Conlan et al., 2017; Da Ros et al., 2022; Osinga et al., 2012). Formulated heterotrophic feeds, which can be tailored to meet species specific nutritional requirements, may provide several advantages over live feeds for large scale coral production yet have received little research attention (Nedovic et al., 2011; Thatcher et al., 2022).

4.2 State of knowledge

4.2.1 Coral nutrition

Despite making up only a small proportion of coral biomass, lipids are the favoured metabolic energy source in scleractinian (reef-building) corals (Conlan et al., 2018; Yamashiro et al., 1999). Corals obtain fatty acids, the main constituent of lipids, from both autotrophy and/or heterotrophy, but can also be provided through de novo synthesis by both the photosymbionts and the coral host (Kabeya et al., 2018; Rocker et al., 2019). Certain fatty acids, especially omega-3 long-chain polyunsaturated fatty acids (n-3 LC PUFA) such as eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), provide well-researched benefits to many aquatic organisms and may enhance coral robustness (Conlan et al., 2017). While live feeds such as *Artemia* and microalgae, in-part, provide these nutrients, they are subject to variable quality and their nutritional profile may not be suited to coral species with a limited ability to capture live feeds (Conlan et al., 2018; Conlan et al., 2019). The development of a suitable 'vehicle' to provide key nutrients for captive corals may improve coral aquaculture and in-turn, support reef restoration efforts. To this end, harnessing microencapsulation technology may be a useful approach.

4.2.2 Microencapsulated diets

Microencapsulation is used across the human food and nutraceutical sectors and has the potential as a viable delivery system for bioactive compounds and immunostimulants in aquaculture (Masoomi Dezfooli et al., 2019; Nedovic et al., 2011). This process protects ingredients from oxidation and the small size of the capsules reduces possible palatability or sensory issues for species with a typically low dietary lipid requirement (Nedovic et al., 2011; Wang et al., 2014). Microencapsulation protects bioactive compounds within various coating or "shell" materials (Nedovic et al., 2011) and can be used to encase essential macro- and micro-nutrients as required by captive corals (**Figure 4**). Therefore, developed feeds can be supplemented with additional nutritional components, for example biologically active fatty acids. The potential of novel microencapsulated feeds, rich in n-3 LC PUFA, to improve captive coral culture and contribute towards reef restoration efforts more broadly, however, remains unexplored. Therefore, a series of experiments were conducted by AIMS and their research partners through the Reef Restoration and Adaptation Program, to test the efficacy for microencapsulated feeds to enhance the nutritional profile, increase the survival and improve the growth performance of captive corals.

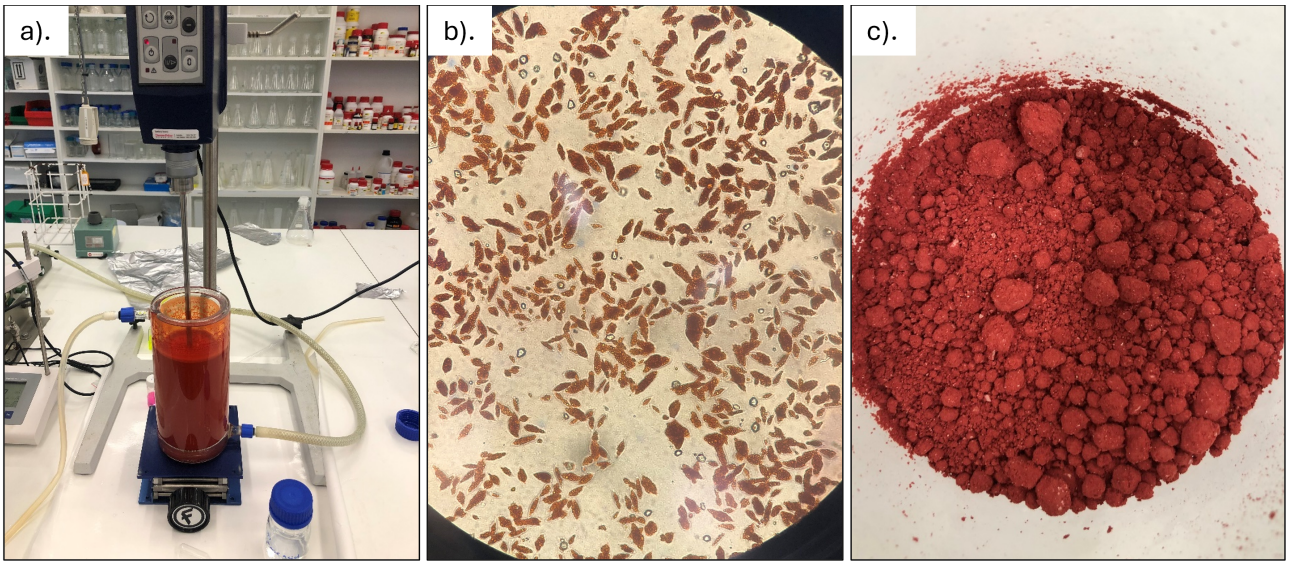

Figure 4*: Production of microencapsulated feed. a). Temperature-controlled mixing of microcapsule solution. b). Microscope image of microcapsule solution following complex coacervation. c). Spray-dried microcapsules. Photo credit: Tom Mock.*

4.3 Approach

Across a series of pilot studies, it was found that microencapsulated feeds are ingested by multiple species of captive corals, as long as the feeds contain an attractant to enhance palatability. Coral species including *A. kenti*, *P. daedalea* and *Pocillopora acuta* showed a positive feeding response toward these novel feeds (Figure 5). Following these promising pilot studies, longer term studies were conducted using *A. kenti*, *Acropora digitifera* and *G. retiformis* juveniles to assess the effectiveness of microencapsulated feeds for coral aquaculture (Figure 6). Particular emphasis has been placed on the effect of formulated microencapsulated feeds on the survival, growth performance and nutritional composition of coral juveniles.

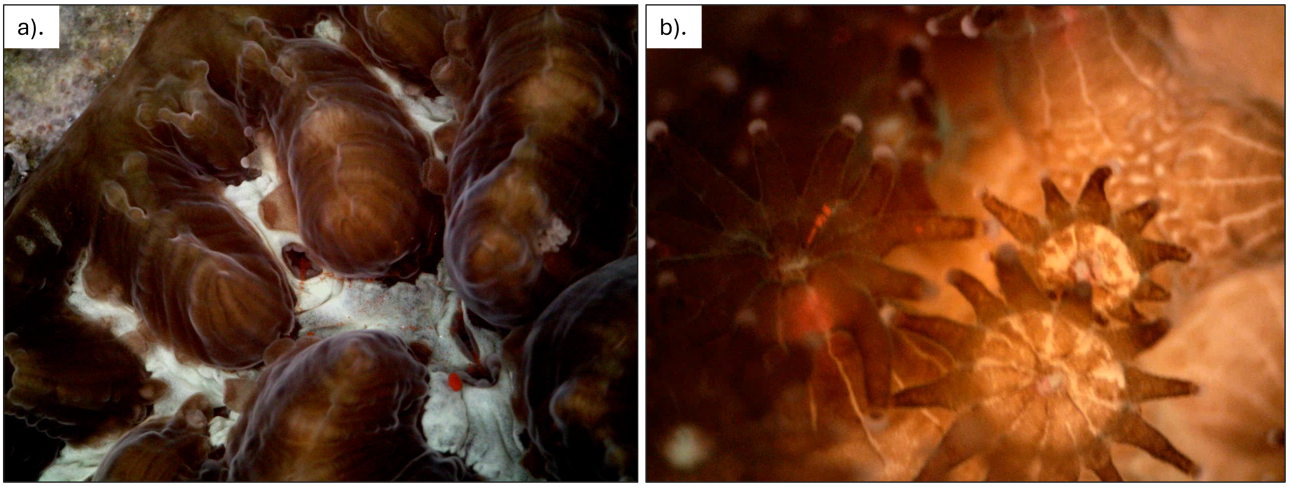

Figure 5*: Ingestion of microencapsulated feeds by a).* Platygyra daedalea *(Photo credit: Stephanie Garra), and b).* Pocillopora acuta (*Photo credit: Alessandro Delli Paoli Carini).*

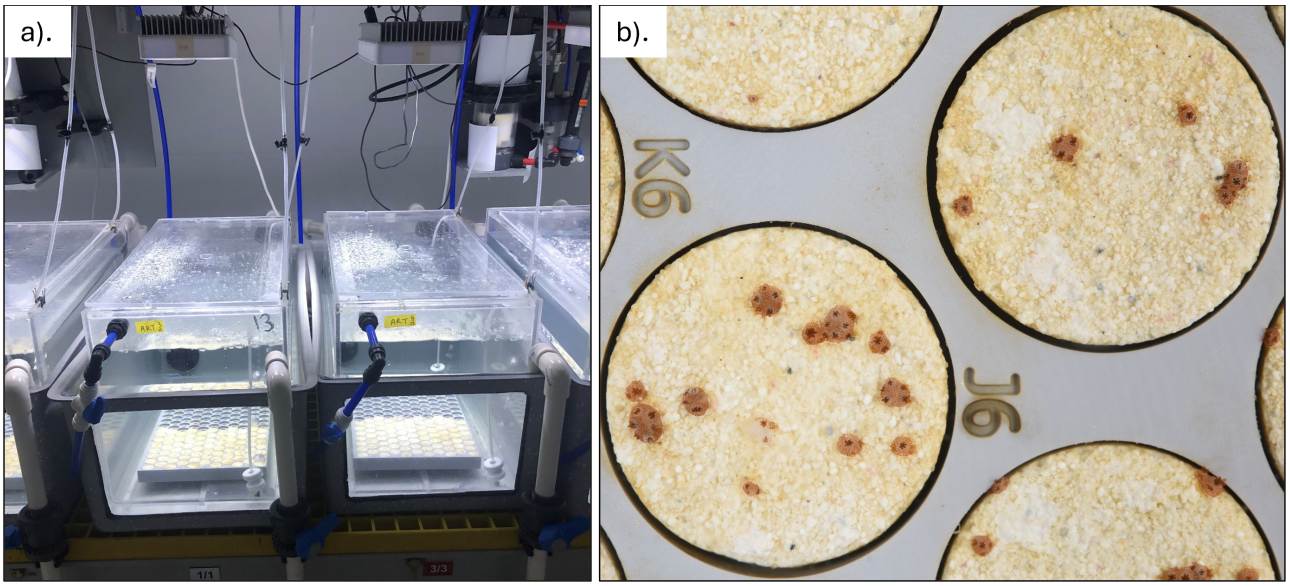

***Figure 6**: Testing microencapsulated feeds for coral aquaculture. a). Experimental setup in 50L flow-through system with coral spat settled on ceramic plugs. Photo credit: Tom Mock. b). 6-week old Acropora kenti juveniles fed with microencapsulated diet. Photo credit: Aman Gosain/Stephanie Garra.*

4.3.1 Survival

Across two 90-day feeding trails, the survival rates of *A. kenti* and *G. retiformis* juveniles fed the microencapsulated feeds was high (>80%) and similar to those fed live feeds and the non-fed controls. These survival rates were higher than previously reported in comparable experiments (Conlan et al., 2017). Interestingly however, a 90-day dose: response experiment where *A. digitifera* juveniles were fed either microencapsulated feeds at increasing doses, *Artemia*, or no feed suggested that coral recruit survival may be enhanced with higher feeding rates of microencapsulated feeds. Across the four capsule dose rates, *A. digitifera* juveniles displayed an increasing trend in survival after 45 days when corals fed the highest dose of microencapsulated diets recorded the highest survival rates (97%). On the other hand, survival rates of juveniles fed *Artemia* were 86% after 45 days, and the survival of unfed *A. digitifera* juveniles was 84%. Trends of survival were however less clear after 90 days.

4.3.2 Growth performance

Across multiple experiments, the growth performance of *A. kenti* (2 experiments) and *G. retiformis* (1 experiment) juveniles fed microencapsulated diets was assessed. In both experiments with *A. kenti*, the growth performance at the conclusion of the 90-days experiments revealed that coral size was similar between corals fed microencapsulated and live feeds. Both live feeds and microencapsulated feeds appeared beneficial compared to unfed corals based on numerically improved performance metrics. However, the experiment with *G. retiformis* showed enhanced performance in treatments supplied with *Artemia* relative to capsules, and a significant growth response to a prolonged period with feed available (identical dose). This is likely due to a limited intake and digestion of the encapsulated diets by this coral species.

4.3.3 Nutritional composition

Detailed nutritional analyses of both *A. kenti* and *G. retiformis* coral juveniles fed live feeds and microencapsulated feeds showed that there was little modulation in their nutritional profile, although there is some evidence that the level of omega-3 and omega-6 fatty acids in coral juveniles may be influenced by this novel feeding strategy. The lipid profiles of reef building corals can fluctuate in response to environmental and nutritional factors as well as symbiont type and diversity (Conlan, 2018; Cooper et al., 2011). However, the extent of change in the nutritional profiles of coral tissue is restricted due to the high

level of genetic control over the lipid and fatty acid profiles of coral species (Imbs & Dembitsky, 2023). In particular, the composition of polar or 'structural lipids', which constitute cell membranes and regulate cell membrane permeability and flexibility are far less susceptible to modification through the provision of formulated feeds compared to non-polar or 'storage lipids' (Cooper et al., 2011; Oku et al., 2003). In agreement with previous research, a large portion of the lipids in the corals in the present experiment were structural lipids meaning the level of nutritional modification due to the formulated feeds was limited. Furthermore, the ratio of storage to structural lipids as well as the total fatty acid profiles in the corals in these experiments did not differ between treatments. Taken together this suggests that the formulated microencapsulated feeds did not significantly alter the nutritional profiles of captive corals compared to the provision of live feeds. Future work could investigate the use of differing doses of microencapsulated feeds given the possibility that higher concentrations may improve survival of coral juveniles. Also, the efficacy of microencapsulated feeds should be assessed for a range of coral species, particularly those with a high requirement for heterotrophically sourced energy and those with a proven capacity for formulated feed ingestion.

4.4 Challenges and opportunities for use in large-scale aquaculture

With the results of the above discussed series of experiments in mind, the following sections detail the opportunities and challenges associated with developing and using microencapsulated feeds for captive corals to embolden reef restoration efforts.

4.4.1 Upscaled production of encapsulated feeds

The microencapsulation of nutrients via complex coacervation at small, experimental scales is both a labour and cost intensive process (Subaşı et al., 2022). Specialised equipment, such as high-speed homogenisers, temperature-controlled vessels and spray drying equipment are required to manufacture microencapsulated feeds and would require significant capital investment. Nevertheless, similar upscaled infrastructure exists to produce encapsulated nutraceuticals and cosmetics for the human food sector and for dietary additives and/or bioactives for the animal feed sector (Arenas-Jal et al., 2020; Gouin, 2004). Therefore, the large-scale production of microencapsulated feeds for captive corals is technically feasible. Clearly, the financial efficacy of large-scale microencapsulated feed production for captive corals would depend on production volume and rely on securing a suitable industry partner for manufacturing and distribution. Nevertheless, if microencapsulated feeds for early life-stage corals could be optimised to provide considerable benefits to coral survivability, growth rates and robustness both before and after deployment, this has the potential to reduce the significant costs associated with captive coral husbandry and deployment efforts

4.4.2 Physical properties of encapsulated feeds

The tested microencapsulated feeds were combined with a fish protein hydrolysate for two reasons: 1) to elicit a feeding response and 2) to increase the density of the feed solution, allowing the corals better access to the feeds by enabling them to sink rather than float on the water surface. While effective, this requires an additional preparation step to combine the microencapsulated powder with the liquid hydrolysate prior to feeding. Further dietary development should investigate the potential to increase the specific gravity of the microencapsulated feed and also the incorporation of a shelf-stable, preferably dry, feed attractant that can be applied to the outside shell of the capsule before feeding.

4.4.3 Incorporation of bioactive compounds

An underexplored application for microencapsulated feeds is their potential to protect and deliver bioactive compounds to captive corals. The benefits of bioactive compounds, such as probiotics to improve the growth, health and disease resistance of other aquatic species has been well-researched (El-Saadony et al., 2021). Microencapsulation may be used to protect potentially sensitive ingredients, such as probiotics from conditions that diminish their viability, such as high temperature, and oxidation (Yao et al., 2020). This

has been successfully applied in feeds for numerous fish and shellfish species (Masoomi Dezfooli et al., 2019) and warrants further investigation in captive corals. If successfully upscaled and adopted by industry partners, the microencapsulation of feed additives and indeed complete feeds would allow access to ready-made off, tailored feed products that remain shelf stable and are therefore able to be deployed on demand.

4.4.4 Biosecurity management

A further avenue that should be explored with respect to coral culture is the potential for formulated feeds, both encapsulated and non-encapsulated to decrease the risk of disease transmission to culture systems. The reliance of live feeds, such as *Artemia*, may introduce pathogens to the culture system, as has been highlighted in the case of shrimp aquaculture (Tacon, 2017). Therefore, the production of formulated feeds, which have typically been subject to high heat, pressure and demoisturisation via oven drying or spray drying may reduce the potential for adverse health outcomes for captive corals.

4.5 Associated risks

Deployment of ex-situ aquaculture produced coral is associated with environmental risks related to possible introduction of pathogens (Sweet et al., 2017), however the use of formulated rather than live feeds could be one way to mitigate this risk (section 4.4.4.). The development of novel feeds could be costly however (Arenas-Jal et al., 2020), and a detailed cost-benefit analysis comparing the use of formulated feeds versus conventional live feeds is needed. Another aspect to consider is that nutrient-dense feeds, if surplus is not efficiently removed from the system, can elevate water nutrient levels (Mock et al., 2019) and stimulate the growth of biofouling algae or opportunistic pathogens, increasing the needs for manual cleaning or co-culture with microherbivores (Neil et al., 2024; Thatcher et al., 2022) The nutritional balance in the system, in particular the N:P ratio, may also influence on the stability of the symbiosis between the coral and Symbiodinaceae (Thatcher et al., 2022).

4.6 Recommendations

The development of formulated feeds optimized for captive corals is expected to support reef restoration efforts. Given the results summarized herein on the use of novel microencapsulated feeds, we recommend that future work include the following activities:

- **Conduct feeding trials with a broader range of coral species.** This would aid in developing feeds tailored to meet species-specific feeding behaviours and nutritional requirements to support upscaled coral aquaculture for restoration of biodiverse reefs.

- **Validate the benefits of feeding coral juveniles by monitoring survival and growth after deployment.** The potential benefits of novel formulated feeds on the survival and growth of cultured corals after deployment are currently unknown.

- **Refine the physical properties of microencapsulated feeds.** The ability to adjust the buoyancy and feed palatability or attractiveness of microencapsulated feeds would enhance the delivery of nutrients to captive corals.

- **Further investigate the potential to upscale the production of microencapsulated feeds.** Feasibility for upscaled treatment production and implementation will depend on securing a suitable industry partner and require a full cost-benefit analysis once further details are known.

5 Conclusions

This report has presented the rationale and approach for three projects addressing bottlenecks in the development of large-scale aquaculture production of coral for use in reef restoration. The projects focused on developing technologies and protocols that can be applied to enhance the settlement success of a range of coral species and to support the survival, health and nutritional status of spat and juveniles, with expected benefits to survival after deployment on the reef.

To reach the goal of large-scale deployment of a diverse range of coral species, advances in settlement-inducing technologies that do not rely on continued environmental collections of CCA is required. Our project identified candidate bacterial inducers of coral settlement and tested their effectiveness across multiple coral species. We confirm that bacterial inducers show potential to overcome challenges related to settlement efficiencies at scale. Future work should prioritise isolation of bacteria-produced cues, test their efficacy in a broader range of coral species, and consider the feasibility of their upscaled production.

Prokaryote-based treatments also offer an opportunity to introduce desirable traits into the coral holobiont and thereby potentially enhancing their health and resilience in the facility and after deployment. Our project generated and tested bacterial probiotic candidates and confirmed that early life stages of coral are amenable to microbiome manipulation through probiotic applications. Future work should pursue the development of single inoculation protocols, confirm host benefits in longer-term studies, and in a broader range of coral species.

Large-scale coral aquaculture may benefit from formulated heterotrophic feeds that can provide controlled and tailored nutrition more consistently than live feeds. We developed and tested palatable microencapsulated diets in a series of coral feeding experiments. A dose-response trial showed that corals exhibited enhanced survival with increasing capsule dose, although further research would be required to establish benefits to growth beyond the use of conventional live feeds. In addition to tailored nutrition, formulated feeds may strengthen biosecurity relative to the use of live feeds, which may introduce pathogens to the system. The development of feeding protocols, diets, and supplements that maintain biosecurity and are easy to implement for both small- and large-scale facilities should be a continued priority.

The treatments described in this report have great promise for improving the efficiency and success of aquaculture production of corals for reef restoration. However, the associated risks of their application to corals that are intended for deployment on the reef would need to be carefully considered. This is currently ongoing via the formal risk assessment process for all restoration interventions considered under RRAP. Furthermore, prioritisation of research areas will need to consider the information that is required to gain regulatory approvals, prior and informed consent from relevant traditional owner groups, and social licence to operate.

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