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Dietary carotenoid supplementation enhances the cutaneous bacterial communities of the critically endangered southern corroboree frog (Pseudophryne corroboree)

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Disciplines

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Dietary Carotenoid Supplementation Enhances the Cutaneous Bacterial Communities of the Critically Endangered Southern Corroboree Frog (*Pseudophryne corroboree*)

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Abstract

The rapid spread of infectious disease has resulted in the decline of animal populations globally. Amphibians support a diversity of microbial symbionts on their skin surface that help to inhibit pathogen colonisation and reduce disease susceptibility and virulence. These cutaneous microbial communities represent an important component of amphibian immune defence; however, very little is known about the environmental factors that influence the cutaneous microbiome. Here, we characterise the cutaneous bacterial communities of a captive colony of the critically endangered Australian southern corroboree frog, *Pseudophyrne corroboree*, and examine the effect of dietary carotenoid supplementation on bacterial abundance, species richness

and community composition. Individuals receiving a carotenoid-supplemented diet exhibited significantly higher bacterial abundance and species richness as well as an altered bacterial community composition compared to individuals that did not receive dietary carotenoids. Our findings suggest that dietary carotenoid supplementation enhances the cutaneous bacteria community of the southern corroboree frog and regulates the presence of bacteria species within the cutaneous microbiome. Our study is the second to demonstrate that carotenoid supplementation can improve amphibian cutaneous bacterial community dynamics, drawing attention to the possibility that dietary manipulation may assist with the ex situ management of endangered species and improve resilience to lethal pathogens such as *Batrachochytrium dendrobatidis* (Bd).

Keywords

Amphibian
Diet
Disease
Innate immunity
Nutrition
Probiotic bacteria

Electronic supplementary material

The online version of this article (doi: 10.1007/s00248-016-0853-2) contains supplementary material, which is available to authorized users.

Introduction

Emerging infectious diseases pose an extreme threat to wildlife biodiversity due to their potential to drive rapid loss in the abundance and genetic composition of animal populations [1]. A prominent cause of amphibian species decline, and extinction has been the rapid emergence and spread of *Batrachochytrium dendrobatidis* (*Bd*), a pathogenic fungus which colonises the skin of amphibians and causes the potentially lethal disease chytridiomycosis [2, 3]. Over the past decade, considerable research attention has focused on understanding and mitigating the threat of *Bd*; however, a long-term solution remains elusive [2]. Of considerable interest, is why certain amphibian species, or populations, persist within *Bd* infected areas, while others do not. Recent research suggests that differences in the amphibian cutaneous microbial community and the function of the mucosome (micro-ecosystem of the skin mucus) may predict relative disease susceptibility [4].

Amphibians are known to support a diverse array of microbial symbionts on their skin surface that help mediate pathogen colonisation [5, 6]. In particular, *Bd* infectivity and virulence is inhibited by antifungal metabolites, such as violacein, which are produced by certain bacteria species [7]. The presence or absence of these anti-*Bd* bacterial species in the skin microbiome has been shown to affect *Bd*-induced amphibian morbidity and mortality [7, 8]. In addition to the influence of bacterial community composition on host resilience to pathogenic infection, increased microbial species richness may contribute to amphibian innate immunity through niche space competition [9]. While evidence for the importance of skin microbiota in amphibian immune defence accumulates, what remains largely unexplored is which abiotic environmental factors experienced by the host amphibian affect these microbial communities [6, 10].

In captivity, restricted exposure to natural environmental bacteria reservoirs has been shown to result in reduced bacterial species richness and divergence in the bacterial community composition of captive amphibians compared to wild conspecifics [11, 12]. These findings suggest that overall immunity of individuals may be compromised while in captivity, which may lead to increased disease susceptibility and reduced survivorship, body condition, and reproductive output both pre- and post-release. A greater understanding of the influence of the captive environment on amphibian skin microbiota is therefore required to enhance the success of ex situ conservation programmes that have been established for endangered amphibians. A key factor known to affect overall health and disease susceptibility of captive amphibians is nutrition reviewed by [13]. To date, however, only one study has investigated the effect of host diet on amphibian cutaneous microbial communities [14], an important component of amphibian immune defence. Antwis et al. [14] investigated the effect of dietary carotenoid enrichment on the cutaneous bacterial communities of captive red-eyed tree frogs, *Agalychnis callidryas*, and found that carotenoids significantly increased bacterial species richness, abundance and overall community composition.

Carotenoids, such as β-carotene, lutein and astaxanthin, are a group of natural pigments biosynthesised by plants and microorganisms, which vertebrates acquire via dietary means [15]. It is well established that carotenoids enhance the immune function of vertebrates through their efficient biological antioxidant activity, sequestering and thereby reducing the harmful effects of reactive oxygen species (ROS) [16]. However, unlike other vertebrates, very little is known about the specific effects of dietary carotenoids on either the innate or adaptive immune system of amphibians. Results reported by Antwis et al. [14]

clearly demonstrate the potential for carotenoid enrichment to enhance the immune function of captive amphibians through their effect on cutaneous bacterial communities. Further research is now required on a diversity of amphibian species in order to ascertain the generality of their findings.

The present study aimed to use bacteria culturing and identification techniques to (1) characterise the cutaneous bacterial community of a captive colony of critically endangered southern corroboree frogs, *Pseudophryne corroboree*, and (2) quantify the effect of dietary carotenoid supplementation on the abundance, species richness and overall community composition of cutaneous bacterial communities.

Material and Methods

Ethics Statement

All procedures outlined in the present study were approved by the University of Wollongong Animal Ethics Committee (Protocol Number: AE13/13).

Study Species

P. corroboree is an endemic Australian frog belonging to the family *Myobatrichidae*. This small (25–30-mm snout-vent length) terrestrial anuran is restricted to fragmented sub-alpine regions of Kosciuszko National Park, New South Wales (1300–1760 m elevation; area of occupancy <0.8 km^2) and is considered critically endangered by the IUCN [17, 18, 19]. The habitat of *P. corroboree* frogs includes wet tussock grasslands, wet heath and adjacent woodlands, with breeding occurring within sphagnum bogs around shallow pools [17, 19]. Average yearly temperatures range from −5 °C to 20 °C (Australian Bureau of Meteorology) with adults entering a state of torpor at the onset of winter [19]. *P. corroboree* typically feeds on algae and organic matter as tadpoles and on ants and other small invertebrates as adults [19, 20, 21]. These dietary items all contain carotenoids [19], so it is assumed that dietary carotenoids form a natural component of the diet of *P. corroboree*.

Experimental Design

To examine the influence of dietary carotenoids on cutaneous bacteria populations, frogs ($n = 44$) were randomly assigned to one of two dietary treatments: (1) an ‘Un-supplemented’ diet whereby frogs ($n = 22$) received a basal control diet with no dietary carotenoid supplementation and (2) a ‘Carotenoid-supplemented’ diet whereby frogs ($n = 22$) received the basal diet supplemented with carotenoids.

Frog Diet and Husbandry

P. corroboree eggs were obtained from a captive colony housed at Melbourne Zoo, Australia. Eggs were stored together on moist sphagnum moss (*Sphagnum cristatum*) in a plastic container (17.5 cm × 12 cm × 7 cm) at 5 °C to suspend embryonic development for approximately 3 months post-fertilisation. Eggs were transferred to the University of Wollongong's Ecological Research Centre on the 19th of July 2013, where they were held in an artificially illuminated constant temperature room set to 12 °C. *P. corroboree* eggs were flooded with reverse-osmosis (RO) water to stimulate hatching, which occurred over 11 days. Immediately after hatching, tadpoles were housed in isolation according to the husbandry procedures stated elsewhere, see [22]. At the commencement of the experimental period (12–18 days after hatching), tadpoles were randomly assigned to either a carotenoid-supplemented diet or an un-supplemented diet. The experimental containers were positioned on three shelves in a constant temperature room, and containers were aligned in rows of three, with the treatment group alternating between rows. Individuals remained in this position and were maintained on experimental diets throughout both juvenile (tadpole) and adult (frog) life-stages.

For the first 8 weeks, un-supplemented tadpoles received two droplets three times a week (range = 0.0585–0.0685 g wet mass, 0.015–0.018 g dry mass) of a thawed and homogenised food solution containing 1.0-g ground fish flakes (25 % san tropical, 75 % flora vegetable flake; SERA®, Germany) suspended in 10-mL RO water. Food supply was then increased to four droplets (range = 0.117–0.137 g wet mass, 0.03–0.036 dry mass) until metamorphosis (forelimb emergence: Gosner stage 42). Carotenoid-supplemented tadpoles received the basal diet described above supplemented with 20-mg carotenoid mixture (Superpig, Repashy Ventures, Inc., Oceanside, CA, USA) per gram of fish flakes. During metamorphosis (Gosner stages 42 to 46), the water volume in each container was reduced from 600 to 150 mL and a small sponge added (9.5 cm × 7.2 cm × 1 cm) to allow for frogs to emerge from the water. Individuals were not provided with food during this stage as nutritional needs are met by reabsorption of the tail.

Upon the completion of metamorphosis (Gosner stage 46), constant room temperature was incrementally increased to 16.5 °C over 14 days. Metamorphs were held in individual plastic containers according to the husbandry procedures described previously, see [22]. Individuals were fed an ad libitum diet of live crickets, *Archea domestica*, twice per week prior to the introduction of experimental diets (1 month after the last frog metamorphosed). Frog body mass did not differ between the treatment groups prior to the introduction of post-

metamorphic experimental diets (*t* test: $t_{43} = 0.842$, $P = 0.403$). Un-supplemented frogs received an ad libitum diet of *A. domestica* twice per week. Crickets were fed on granny smith apples (skin-removed) 48 h prior to feeding. Carotenoid-supplemented frogs also received an ad libitum diet of *A. domestica* twice per week. These crickets were fed only carrot 48 h prior to feeding and dusted with 1.0 g carotenoid mixture prior to feeding. Once a week, crickets in both diet treatments were dusted with 0.2 g of calcium powder prior to feeding (Repti-Cal, Aristopet, Australia). Crickets ranged in age from 2 to 10 days for both dietary treatments, and their size was increased consistently as frogs increased in size. When frogs were approximately 87 weeks of age post-hatching, the temperature was incrementally increased (over 2 weeks) to 20 °C, 6 weeks prior to the start of our experiment.

Bacteria Sample Collection

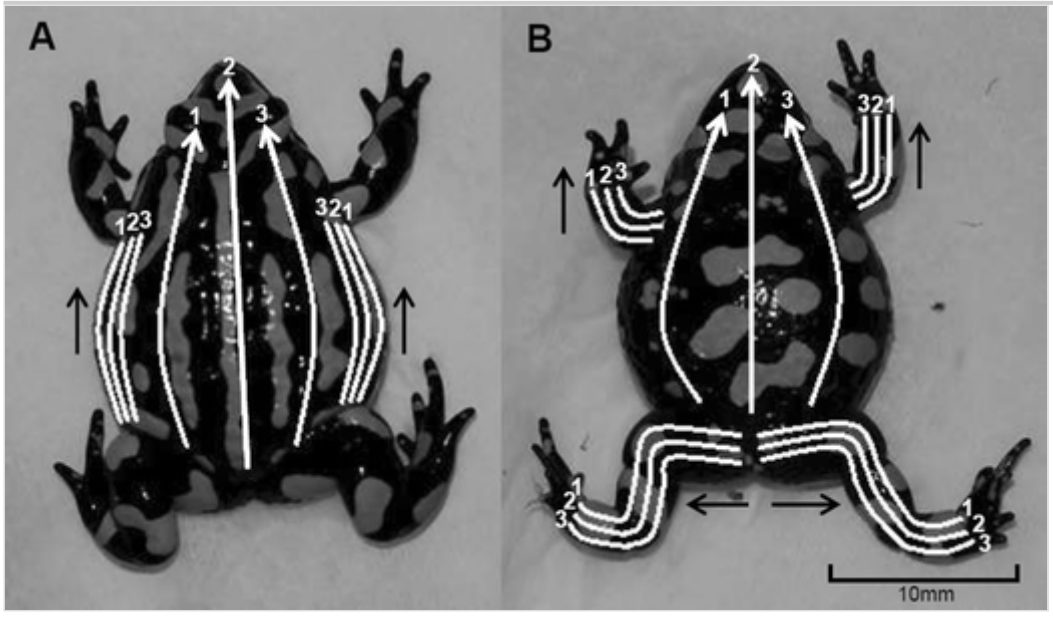
Bacteria samples were collected over three sampling days, the 25/05/2015 ($n = 5$ frogs per treatment), 01/06/2015 ($n = 7$ per treatment) and 08/06/2015 ($n = 10$ per treatment). Each individual was swabbed only once. At the commencement of the sampling period, frogs were approximately 96 weeks of age post-hatching. After swabs were collected, each frog was weighed and photographed (Samsung ST66 16.1 megapixel camera, Samsung Group, South Korea) on top of sterile laboratory tissue (Kimtech, Kimberly-Clark, Roswell, GA, USA), which was replaced between individuals. Subsequent length measurements were made from digital images using ImageJ software (National Institute of Health, USA). Frog mass (range = 1.73–2.80 g; mean = 2.18 ± 0.04 g) and snout-vent length (range = 23.46–27.51 mm; mean = 25.52 ± 0.20 mm) did not differ between supplemented and un-supplemented treatments ($n = 22$ per treatment); *t* test: weight: $t_{43} = 1.30$, $P = 0.20$; snout-vent: $t_{43} = 0.99$, $P = 0.33$). During swab collection, frogs were removed from their individual containers and handled with separate sterile gloves (Skinshield powder-free latex gloves, Livingstone International, Rosebery, NSW, Australia) to prevent cross contamination of cutaneous bacteria [14, 23]. Immediately prior to sampling, frogs were rinsed once with 30-mL sterile water to remove transient bacteria [6, 23, 24], ensuring minimal handling to prevent loss of cutaneous bacteria.

Cutaneous bacteria samples were collected using sterile transport swabs (Copan Transystem® Amies agar gel medium without charcoal, Copan Diagnostics, Inc., USA). Frogs were swabbed (1 swab/frog) by running the natural fibre cotton tip across each of the following surfaces three times: dorsal (anterior to posterior), ventral (anterior to posterior), lateral (left and right sides), front legs from armpit to wrist (left and right sides) and back legs from groin to ankle (left and right sides) (adapted from Shaw et al. [24] and Antwis et al. [14] (Fig. 1). Rotation

of the swab throughout sample collection promoted even bacterial coverage on the swab. Labelled swabs were placed in storage tubes containing sterile transport media and stored on ice for 150 ± 30 min (storage temp = 5.5 ± 0.6 °C) during transfer to the lab for processing.

Fig. 1

Cutaneous bacteria swabbing protocol. *White lines* represent body regions swabbed on the **a** dorsal and **b** ventral surfaces of each frog (1 swab/frog). Each region was swabbed in triplicate (numbering 1, 2 and 3). *Arrows* represent swabbing direction



Bacterial Culture and Identification

Using aseptic techniques within a biosafety cabinet (Biological Safety Cabinet Class II, Email Westinghouse Pty Ltd, Australia), swab tips were cut off into a 1.5-mL sterile Eppendorf tube (Eppendorf South Pacific Pty Ltd, North Ryde, NSW, Australia) containing 1 mL 0.9 % sterile NaCl and vortexed for 30 s to disassociate bacteria from the tip [14]. As indicated by a pilot study, dilutions of 10^{1} (undiluted) and 10^{-1} (10-fold dilution) in 0.9 % sterile NaCl were most appropriate to obtain an optimum amount of bacterial growth suitable for assessing bacterial community composition (i.e. 25–300 colony-forming units (CFU); [25] when compared with 10^{-2} and 10^{-3} dilutions. A 10^{-1} dilution was prepared by pipetting 100 µL of the undiluted solution into 900 µL 0.9 % sterile NaCl. All solutions were vortexed for further 5 s immediately before dilutions and plating to ensure a homogenous bacterial suspension.

Each swab was plated in triplicate (1×10^{1} undiluted solution and 2×10^{-1} dilution) by transferring 100-µL aliquot of bacterial suspension to an LB (Luria Broth) nutrient agar media plate (100-mm diameter; AMRESCO®, Solon, OH,

USA) and spread evenly over the surface using a sterile, stainless steel plate spreader (35-mm blade, 4-mm rod; Ja Hely, Zhejiang, China). Equipment was sterilised using an autoclave (Model HS5510EC-1; Getinge, Murarrie, Australia). Plates were dried for 10 min prior to being inverted and sealed with parafilm M® laboratory film (Pechiney Plastic Packaging, Chicago, USA). Plates were incubated for 72 ± 1 h at 20 °C. This incubation temperature was chosen based on the outcome of a pilot study showing that *P. corroboree* cutaneous bacteria growth ($n = 6$ plates per treatment) at an incubation temperature of 20 °C had significantly higher bacterial species richness, bacterial abundance and optimal time-efficiency of growth in comparison with 5, 10 and 30 °C (see Supplementary Material S1). This is also the temperature at which the captive frogs were maintained throughout the experimental period.

At 72 ± 1 h, plates were photographed (Samsung ST66 16.1 megapixel camera, Samsung Group, South Korea) for analysis in ImageJ (National Institute of Health, USA) to obtain total CFU counts. An incubation time of 72 h was found to be most appropriate for obtaining an accurate CFU count (incubation at 20 °C) while maintaining maximum time efficiency (see Supplementary Material S1). The most appropriate plate from each triplicate was selected on the basis of total CFU range within 25–300 CFU [25]. If more than one plate per frog was suitable, the plate used was randomly selected. CFU counts were standardised using the dilution factor of each plate to obtain CFU/mL of suspension solution.

Plates continued to be incubated at 20 °C for a total of 168 ± 24 h to ensure sufficient time for the development of distinct colony morphology characteristics, and where appropriate, plates were transferred to 4 °C to prevent overgrowth of fungi prior to species isolation. All morphologically distinct colonies within each triplicate of dilution plates were allocated an isolate ID number. These were transferred to a separate LB nutrient agar plate using standard 16-streak method to obtain pure cultures and a bacteria species richness count for each frog [26]. Isolate plates were compared across frogs and morphologically distinct isolate plates were grouped and assigned a morphotype ID number. One representative from each morphotype ID was processed for identification.

Bacteria identification was conducted using bioMérieux API® 20E™ identification system (bioMérieux Australia Pty Ltd, Baulkham Hills, Australia) and apiweb™ identification software (bioMérieux Australia Pty Ltd, Baulkham Hills, Australia). This identification system is limited to Enterobacteriaceae and non-fastidious gram-negative species. Species that could not be identified using this system ($n = 8$) were identified using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS)

(Bruker Daltonik MALDI Biotyper, Bruker Biosciences, Victoria, Australia) at Pathology North (Department of Microbiology, Royal North Shore Hospital, St. Leonards, Australia). MALDI-TOF MS is a highly accurate method of identification for a broad spectrum of bacteria, including gram-positive cocci and rods and fermentative and non-fermentative gram-negative rods [27]. Bacteria isolates that did not provide a match in identification databases were assigned a letter and accompanied by a detailed morphotype description (see Supplementary Material S2).

Statistical Analyses

Two separate ANOVA models were used to determine whether there were any temporal effects of sampling frogs on three different days. No temporal effects were detected on either cutaneous bacterial abundance ($F_{2, 41} = 1.20$, $P = 0.31$) or species richness ($F_{2, 41} = 0.69$, $P = 0.51$), and so sampling days was not added as a factor in any subsequent analyses. To determine the effect of carotenoid supplementation on amphibian cutaneous bacteria abundance, and bacteria species richness, two independent *t* test models were conducted using JMP 10® statistical software. To correct for multiple comparisons, a Bonferroni correction was applied, reducing the critical *P* value to 0.025 for each test. Prior to running the *t* tests, assumptions of the model were tested using Shapiro-Wilk's normality test and the Levene's test for homoscedasticity. Bacterial count data (CFU) were subsequently log transformed.

A bacteria species presence/absence data set was created to allow for the comparison of overall bacterial community composition between dietary treatments, which was performed using multivariate analysis in PRIMER 7. A Bray Curtis similarity index and multi-dimensional scaling (nMDS) ordination was produced to allow for a visual comparison of treatment-related patterns before proceeding with further analyses. Differences in overall community composition of cutaneous bacteria between treatments were analysed using PERMANOVA. SIMPER analysis was used to determine which bacteria were most influential in determining differences between bacteria assemblages of frogs in each diet treatment. Data were from random, independent samples. Bacteria species assemblage data was compiled to produce a list of species present within the different dietary treatments and a bacteria family-level comparison of diet treatments.

Results

Dietary carotenoid supplementation had a significant effect on total cutaneous bacterial abundance (*t* test: $t_{43} = 2.77584$, $P = 0.0053$, Bonferroni-corrected),

with frogs fed a carotenoid-supplemented diet supporting a significantly greater bacterial abundance than frogs fed on an un-supplemented diet (Fig. 2). Overall, bacterial abundance was not correlated with frog mass ($F_{1, 42} = 0.0120$, $P = 0.9133$, $r^2 = 0.000261$) or snout-vent length ($F_{1, 42} = 1.2027$, $P = 0.2785$, $r^2 = 0.025479$) Dietary treatment also had a significant effect on bacterial species richness (*t* test: $t_{43} = 3.57369$, $P = 0.0005$, Bonferroni-corrected; Fig. 3), with frogs that received a carotenoid-supplemented diet supporting a greater number of cutaneous bacteria species compared with frogs that received an un-supplemented diet (Fig. 3). Overall, species richness was not correlated with frog mass ($F_{1, 42} = 1.1757$, $P = 0.2839$, $r^2 = 0.000261$) or snout-vent length ($F_{1, 42} = 0.0120$, $P = 0.9133$, $r^2 = 0.025479$). Regression analysis also confirmed there was no correlation between bacterial abundance and species richness (regression: $F_{1, 42} = 0.0091$, $P = 0.9243$, $r^2 = 0.0002$).

Fig. 2

Abundance (number of colony-forming units (CFU) per mL) of isolated from carotenoid-supplemented and un-supplemented *P. corroboree* frogs ($n = 22$ per treatment). Data shown are mean ± S.E.M. An *asterisk* denotes a significant difference between treatments ($P < 0.05$)

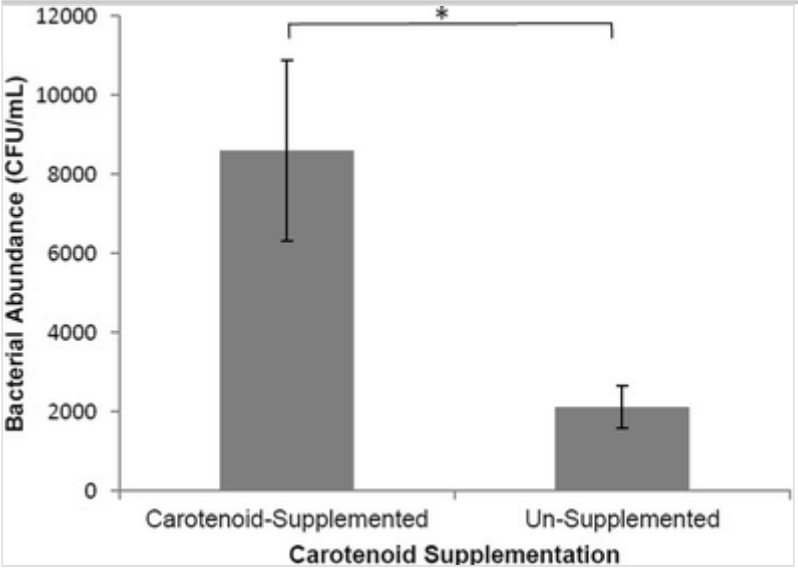
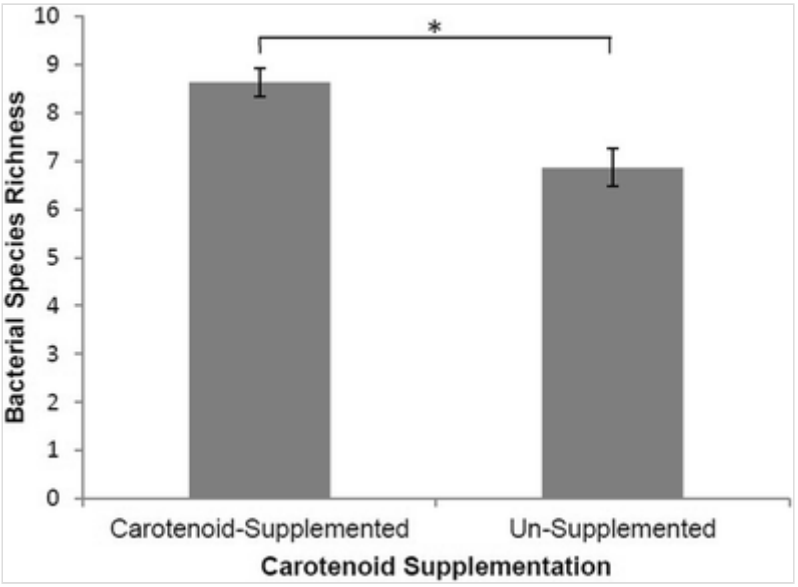


Fig. 3

Species richness of bacteria isolated from carotenoid-supplemented and un-supplemented *P. corroboree* frogs ($n = 22$ per treatment). Data shown are mean ± S.E.M. An *asterisk* denotes a significant difference between treatments ($P < 0.05$)



A total of 23 bacterial species were isolated from captive *P. corroboree* (Table 1). Twenty-two of these bacterial species were present on *P. corroboree* fed a carotenoid-supplemented diet, and 20 were present on *P. corroboree* fed an un-supplemented diet (see Supplementary Material S3). In total, 83 % of bacteria species were found across both treatments, with species from the family Moraxellaceae completely absent from un-supplemented frogs (Fig. 4). Three bacteria species (*Escherichia coli*, *Acinetobacter calcoaceticus* and Unidentified C) were isolated from the skin of carotenoid-supplemented individuals but were not present on un-supplemented individuals. One species, Unidentified E, was only present on the skin of un-supplemented individuals (Table 1).

Table 1

Bacteria species isolated from captive *P. corroboree* fed on a carotenoid-supplemented diet and an un-supplemented diet ($n = 22$ per treatment)

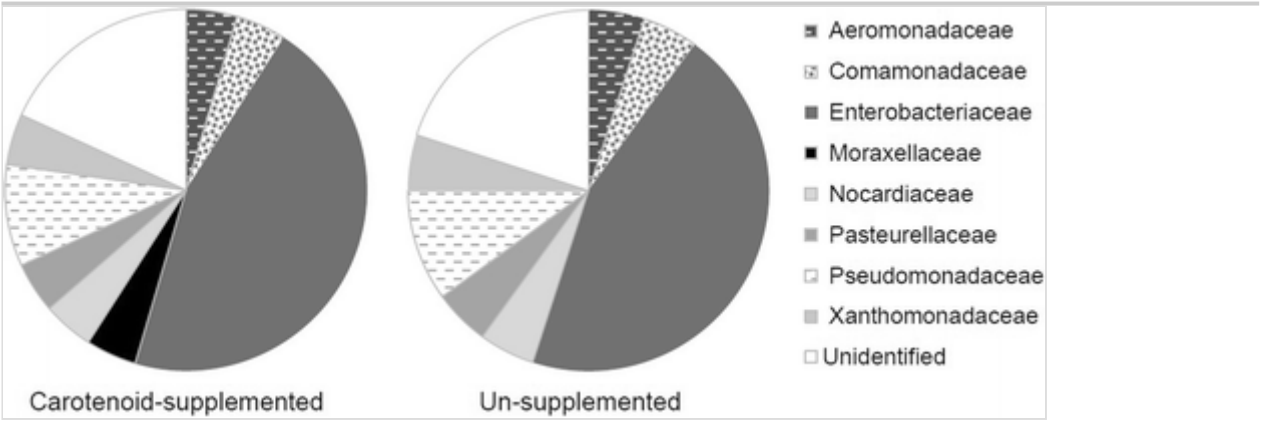
Family	**Species (APIWeb % match)**	**Percentage occurrence of bacteria species in captive *P. corroboree* (the number in brackets represents the number of frogs with bacteria species present).**	
		Carotenoid-supplemented frogs	**Un-supplemented frogs**
Aeromonadaceae	*Aeromona hydrophila* (80 %)	18.2 (4)	36.4 (8)
Comamonadaceae	*Variovorax paradoxus*[a]	59.1 (13)	45.5 (10)
Enterobacteriaceae	*Citrobacter braakii* (99.3)	40.9 (9)	54.5 (12)

Enterobacteriaceae	*Citrobacter freundii* (99.8 %)	9.1 (2)	18.2 (4)
Enterobacteriaceae	*Escherichia coli* (87 %)	4.5 (1)	0 (0)
Enterobacteriaceae	*Ewingella americana*[a]	45.5 (10)	13.6 (3)
Enterobacteriaceae	*Klebsiella oxytoca* (99 %)	36.4 (8)	27.3 (6)
Enterobacteriaceae	*Klebsiella pneumoniae spp* (99.9 %)	18.2 (4)	4.5 (1)
Enterobacteriaceae	*Morganella morganii* (98 %)	72.7 (16)	77.3 (17)
Enterobacteriaceae	*Proteus mirabilis* (89.9 %)	59.1 (13)	50 (11)
Enterobacteriaceae	*Serratia marcescens* (96 %)	4.5 (1)	9.1 (2)
Enterobacteriaceae	*Serratia sp* (92 %)	90.9 (20)	54.5 (12)
Moraxellaceae	*Acinetobacter calcoaceticus* (99 %)	4.5 (1)	0 (0)
Nocardiaceae	*Rhodococcus erythopolis*[a]	45.5 (10)	18.2 (4)
Pasteurellaceae	*Pasteurella pneumotropica* (87 %)	9.1 (2)	4.5 (1)
Pseudomonadaceae	*Pseudomonas luteola* (91 %)	68.2 (15)	27.3 (6)
Pseudomonadaceae	*Pseudomonas oryzihabitans* (87.3 %)	4.5 (1)	9.1 (2)
Xanthomonadaceae	*Stenotrophomonas maltophilia* (99.3 %)	100 (22)	100 (22)
	Unidentified A	54.5 (12)	31.8 (7)
	Unidentified B	63.6 (14)	59.1 (13)
	Unidentified C	36.4 (8)	0 (0)
	Unidentified D	18.2 (4)	13.6 (3)
	Unidentified E	0 (0)	31.8 (7)

[a]Species without API% match were identified using MALDI-TOF

Fig. 4

Relative occurrence of bacteria phyla identified from carotenoid-supplemented and un-supplemented *P. corroboree* frogs ($n = 22$ per treatment)



Overall, the community composition of cutaneous bacteria differed significantly between frogs fed on a carotenoid-supplemented diet and those fed an un-supplemented diet (PERMANOVA: Pseudo-$F_{1, 46} = 3.6754$, P (perm) = 0.003). However, the two treatment groups had a relatively low average dissimilarity of 48.92 %. *Pseudomonas luteola* contributed most to species assemblage differences (7.53 % contribution of total dissimilarity) and was significantly more common within the dietary carotenoid supplementation treatment ($\chi^2_1 = 29.673$, $P < 0.0001$). Another four species that were significantly more common within the dietary carotenoid supplementation treatment group were as follows: *Ewingella americana* (% contribution = 6.76, $\chi^2_1 = 25.259$, $P < 0.0001$); Unidentified A (% contribution = 6.39, $\chi^2_1 = 13.032$, $P = 0.0003$); *Rhodococcus erythopolis* (% contribution = 6.25, $\chi^2_1 = 20.051$, $P < 0.0001$) and *Serratia sp*. (% contribution = 5.76, $\chi^2_1 = 33.122$, $P < 0.0001$). Further, species were not assessed for differences between treatments as percentage contribution to treatment dissimilarity was less than 5.5 %, which was considered low. No contributing species (>5.5 % contribution) occurred more commonly within the un-supplemented treatment. *Stenotrophomonas maltophilia* was the most commonly occurring species, being present on all individuals and contributing 19.53 % to total treatment similarity.

Discussion

Carotenoids are well recognised for their ability to enhance vertebrate immune function [16]. However, very little is known about the influence of dietary carotenoids on amphibian cutaneous microbiota, despite the importance of these bacterial communities for amphibian immune defence [14]. Here, we quantified the effect of dietary carotenoid supplementation on the abundance, species

richness and overall community composition of the cutaneous bacterial communities of the critically endangered southern corroboree frog, *P. corroboree*. As predicted, frogs that received a carotenoid-supplemented diet exhibited significantly higher cutaneous bacterial abundance and species richness compared with individuals that did not receive dietary carotenoids. Overall, the composition of cutaneous bacterial communities also differed among carotenoid-supplemented and un-supplemented individuals, suggesting that dietary carotenoids play a role in determining which bacteria reside within the cutaneous microhabitat.

While the specific mechanisms by which dietary carotenoids alter the hosts' cutaneous bacterial community currently remain unknown, a number of possible mechanisms could be in operation. First, the enhanced bacterial community attributes reported may reflect a more suitable cutaneous substrate for bacterial colonisation. It has been suggested that the utilisation of carotenoids by the host amphibian may enhance the production of mucous by dermal mucous glands, altering the quantity, nutritional composition or pH of mucous layer to allow the colonisation of a greater abundance or diversity of bacteria [14]. Alternatively, differences in the cutaneous bacterial communities observed may be a result of variation in the secretion of antimicrobial peptides (AMPs) by the host amphibian. AMPs are known to exhibit selective antimicrobial discretion [28], and carotenoid-supplemented frogs may produce peptides that selectively inhibit certain competitively superior bacteria species and allow colonisation by a greater diversity of species. This explanation seems insufficient to explain the results reported in the present study, however, as *P. corroboree* shows low peptide activity compared to other anuran species [29]. Finally, the enhanced microbial communities identified may be a result of the direct utilisation of carotenoids as antioxidants by bacterial cells. Aerobic bacteria continuously generate reactive oxygen species and are known to benefit from antioxidants (such as carotenoids) as a defence against the damaging effects of oxidative stress [30].

Regardless of the mechanism by which cutaneous bacterial communities are enhanced by supplementing the host-amphibians' diet with carotenoids, the outcome could have important implications for the disease resistance of amphibians. Within the captive environment, restricted exposure to environmental reservoirs of bacteria and rigorous hygiene protocols means that the diversity of cutaneous bacteria in captive frogs is typically lower compared to the diversity reported for wild conspecifics [11, 12]. Reduced diversity in cutaneous bacterial communities is known to compromise amphibian innate immune response. For example, recent research found that a reduction in cutaneous microbial richness of redback salamanders, *Plethodon cinereus*,

resulted in enhanced symptoms of chytridiomycosis (the disease caused by Bd) including decreased body mass and increased skin-shedding and limb-lifting behaviour [31]. As such, providing amphibians with captive environmental conditions which promote the diversity and abundance of cutaneous bacteria may be fundamental to improving the immune function and health of captive amphibians. The results of the present study show dietary carotenoid supplementation in captive *P. corroboree* enhanced cutaneous bacterial diversity and abundance. These enhanced bacterial community attributes could act to aid amphibian immunity by leaving less niche space and/or adhesion sites available to invading pathogens, including Bd [9, 31]. Additionally, pathogen colonisation may be reduced on amphibians supporting greater cutaneous bacterial abundance and richness due to the presence of competitively superior bacteria species capable of the secretion of antimicrobial compounds, known as anti-Bd species [31, 32].

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Anti-Bd bacteria species reside within the cutaneous microbiome and are capable of producing antifungal metabolites that target Bd, inhibiting its colonisation and growth [32]. One example is the cutaneous bacterium *Janthinobacterium lividum*, which produces the antifungal metabolite violacein, reported to inhibit Bd growth in lab assays [33, 34]. Furthermore, bioaugmentation experiments with two amphibian species, *Plethodon cinereus* [7] and *Rana mucosa* [7], have demonstrated that violacein effects Bd infectivity and virulence when individuals are inoculated with Bd at low concentrations. Of the 23 bacteria identified in the present study, three bacteria species (*S. maltophilia*, *Serratia marcescens* and *Citrobacter freundii*) are currently known to show Bd inhibitory properties [8, 35, 36]. Across both dietary treatments (carotenoid-supplemented and unsupplemented), all frogs possessed at least one known anti-Bd bacteria species, although the anti-Bd capacity of individuals remains to be tested. It is also important to note that identifying bacteria with anti-Bd properties is the focus of ongoing investigation and additional species are likely to be identified in the near future. It is therefore currently difficult to predict whether carotenoid-supplemented frogs in the present study would secrete greater concentrations of antimicrobial metabolites compared to unsupplemented individuals, and identifying and quantifying these secretions should be the subject of further investigation.

The present study is the second to demonstrate that ex situ dietary supplementation can be used to manipulate amphibian cutaneous bacteria communities. Our results are consistent with those of a recent study on captive red-eyed tree frogs, *A. callidryas*, which reported improved bacterial species richness, abundance and community composition of frogs fed a carotenoid-

enriched diet post-metamorphosis [14]. Taken together, these results suggest that dietary manipulation may enhance innate immune function in amphibians. It is important to note, however, that both studies only identified culturable bacteria and further investigation using culture-independent assays (e.g. Illumina 16s sequencing methods) are now required to better understand the true diversity of bacteria within these cutaneous communities [14]. Additional research is also urgently needed to test how the differences in cutaneous bacterial communities observed as a result of dietary carotenoid supplementation influence disease resistance in endangered amphibians. A recent study by Cothran et al. [37] reported no mitigating effects of dietary carotenoids against Bd infection in two common amphibian species, *Lithobates sylvaticus* and *Hyla versicolor*. However, this study did not consider cutaneous microbial dynamics, so it is unclear whether ineffective pathogen resistance in these two species was a consequence of deficient microbial community dynamics, such as the complete absence of effective anti-Bd bacteria [36]. Interestingly, Cothran et al. [37] also reported reduced growth rates and survivorship of individuals fed a carotenoid-supplemented diet, indicating that the dose administered may have been detrimental to these anuran species. Of note, results from our laboratory show no significant effect of carotenoid supplementation on juvenile or post-metamorphic growth, development or survival of *P. corroboree* (Byrne and Silla unpublished data). Additionally, supplementing southern corroboree frogs with the carotenoid dose used in the present study has recently been shown to enhance colouration and escape performance [22, 38]. Given the positive effects of this concentration of dietary carotenoids on other fitness determining traits, we predict that the enhanced cutaneous bacterial communities reported will translate to increased immunity in this species. Directly testing these effects in *P. corroboree* and other anuran species should be the subject of further investigation.

In conclusion, the results of our study provide evidence that dietary carotenoid supplementation enhances the cutaneous bacteria communities of the southern corroboree frog. We highlight the potential for dietary supplementation of the host amphibian to be used as a tool to manipulate cutaneous bacterial community dynamics, which may have broad implications for amphibian conservation. Specifically, manipulation of cutaneous bacterial communities through dietary control has the potential to improve amphibian innate immunity and enhance their resilience to lethal pathogens such as *Batrachochytrium dendrobatidis* (Bd). Although, studies directly testing the effect of dietary carotenoid supplementation on Bd resistance are now required. Ongoing research exploring relationships between amphibian nutrition, cutaneous microbiota and immunity stands to enhance the success of ex situ and in situ conservation programmes for endangered amphibians.

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Authors' Contributions AS and PB conceived the experiment. AS, PB and PH developed the methods. AS collected the skin swabs. CE collected the data. CE analysed the data with assistance from PB. AS and CE wrote the paper.

Compliance with Ethical Standards

Ethics Statement

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All procedures outlined in the present study were approved by the University of Wollongong Animal Ethics Committee (Protocol Number: AE13/13).

Electronic supplementary material

Below is the link to the electronic supplementary material.

Table S1

Results of pilot study determining optimal incubation temperature for culturing cutaneous bacteria. Data shown are bacteria species richness untransformed mean ± SEM ($n = 6$ per treatment) and bacterial abundance (CFU/plate) untransformed mean ± SEM ($n = 6$ per treatment). The result of a one-way ANOVA is shown by $F_{\text{d.f.}}$ and P values. Letters attached to each mean denote significant differences ($P < 0.05$) between dilution treatments as a result of a post hoc Tukey-Kramer HSD. (DOCX 16 kb)

Table S2

Unidentified bacteria species descriptions. (DOCX 14 kb)

Table S3

Bacteria species assemblages of individual carotenoid-supplemented ($n = 22$) and un-supplemented ($n = 22$) *P. corroboree* frogs. (DOCX 36 kb)

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