Received: 3 December 2021 | Revised: 23 January 2022 | Accepted: 28 January 2022

DOI: 10.1111/wrr.13003

ORIGINAL ARTICLE-REGENERATION SCIENCE

Wound Repair and Regeneration — WILEY

Manipulating the microbiome alters regenerative outcomes in *Xenopus laevis* tadpoles via lipopolysaccharide signalling

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Funding information
Royal Society of New Zealand Marsden Fund, Grant/Award Number: UOO1910; University of Otago; the Royal Society of New Zealand Marsden Fund

Abstract

Xenopus laevis tadpoles can regenerate functional tails, containing the spinal cord, notochord, muscle, fin, blood vessels and nerves, except for a brief refractory period at around 1 week of age. At this stage, amputation of the tadpole's tail may either result in scarless wound healing or the activation of a regeneration programme, which replaces the lost tissues. We recently demonstrated a link between bacterial lipopolysaccharides and successful tail regeneration in refractory stage tadpoles and proposed that this could result from lipopolysaccharides binding to Toll-like receptor 4 (TLR4). Here, we have used 16S rRNA sequencing to show that the tadpole skin microbiome is highly variable between sibships and that the community can be altered by raising embryos in the antibiotic gentamicin. Six Gram-negative genera, including *Delftia and Chryseobacterium*, were over-represented in tadpoles that underwent tail regeneration. Lipopolysaccharides purified from a commensal *Chryseobacterium spp. XDS4*, an exogenous *Delftia spp*. or *Escherichia coli*, could significantly increase the number of antibiotic-raised tadpoles that attempted regeneration. Conversely, the quality of regeneration was impaired in native-raised tadpoles exposed to the antagonistic lipopolysaccharide of *Rhodobacter sphaeroides*. Editing TLR4 using CRISPR/Cas9 also reduced regeneration quality, but not quantity, at the level of the cohort. However, we found that the editing level of individual tadpoles was a poor predictor of regenerative outcome. In conclusion, our results suggest that variable regeneration in refractory stage tadpoles depends at least in part on the skin

Abbreviations: ANI, average nucleotide identity; ANOVA, analysis of variance; bp, base pairs; C, celsius; CaCl, calcium chloride; Cas9, CRISPR-associated protein 9; CD14, cluster of differentiation 14 protein; CFU, colony-forming units; CRISPR, clustered regularly interspaced short palindromic repeats; DAMP, damage-associated molecular pathway; DECODR, deconvolution of complex DNA repair; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; F0, parental generation; F1, first filial generation; FR, full regeneration; HCG, human chorionic gonadotropin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSP60, heat-shock protein (60 kDa); ICMP, international Collection of Microorganisms from Plants; IDT, integrated DNA Technologies; Indel, insertion/deletion; KCl, potassium chloride; LB, Luria broth; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; MD2, myeloid differentiation factor 2; MgCl, magnesium chloride; MgSO4, magnesium sulphate; MMR, Marc's modified ringer's solution; MS222, tricaine methanesulfonate; MyD88, myeloid differentiation primary response 88; NaCl, sodium chloride; NCBI CDD, national center for biotechnology information—conserved domain database; NEB, new England Biolabs; NLS, nuclear localisation signal; NR, no regeneration; NS, not significant; OTU, operational taxonomic unit; PAMP, pathogen-associated molecular pathway; PB, partial bad regeneration; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PC2, physical containment level 2; PG, partial good regeneration; PNA, peptide nucleic acid; RNA, ribonucleic acid; ROC, regeneration-organising cells; rRNA, ribosomal ribonucleic acid; RS-LPS, *Rhodobacter sphaeroides* lipopolysaccharide; sgRNA, single-guide ribonucleic acid; TE, tris-Ethylenediaminetetraacetic acid; TGFb1, transforming growth factor beta 1; TIDE, tracking of Indels by Decomposition (program); TIR, toll-interleukin-1 inhibition domain; TLR, toll-like receptor (TLR2 and TLR4); TM, transmembrane domain; TMHMM, prediction of transmembrane helices in proteins - server; w/v, weight/volume.

Xochitl C. Morgan and Caroline W. Beck are contributed equally to this study.

microbiome and lipopolysaccharide signalling, but that signalling via TLR4 cannot account for all of this effect.

1 | INTRODUCTION

Tadpole tail regeneration in *Xenopus laevis* provides a useful model to study regenerative mechanisms in complex tissues. Tails contain midline neural tube—the forerunner of the spinal cord—as well as notochord, paraxial muscles (somites), blood vessels, nerves and the dorsal and ventral fins (extensions of the epidermis). *Xenopus laevis* is a well-used model organism, and development has been classified into 66 stages, with pre-feeding stages that are well synchronised.[1] Tails regenerate well following partial amputation from stage 40 to 44. From stage 45 to 47, there is a dramatic reduction in the number of tadpoles undergoing regeneration, with a scarless wound healing programme replacing this in many tadpoles.[2] We refer to this as the refractory period, and it is useful since it offers the opportunity for both gain and loss of function experiments in a single system. Prior studies have implicated many developmental signalling pathways, as well as processes such as apoptosis, epigenetic regulation, membrane depolarisation, extracellular matrix remodelling, reactive oxygen species production, inflammatory response and metabolic reprogramming in *Xenopus* tail regeneration (for recent review, see Phipps et al.[3]).

Tails in the refractory period seem to commit to either regeneration or wound healing pathways in the first 6 h following amputation.[2] Tails that successfully recruit regeneration-organising cells (ROCs) to the wound site to form a wound epithelium will go on to organise the regeneration of either fully patterned or pattern-deficient tails[4] via recruitment of underlying distal cells to a regeneration bud.[5] Tails that instead heal with a full-thickness epidermis, including a basement membrane, will not regenerate and do not form a regeneration bud.[6] In many regeneration competent model organisms, macrophages (phagocytic cells that form part of the innate immune system) are critical for regeneration. This is true of zebrafish tails[7,8] and axolotl limbs and heart,[9,10] as well as *Xenopus* tadpole tails.[11] Recent work from our laboratory has shown that the base rate of tadpole tail regeneration is innately variable, with some sibships showing naturally higher regenerative rates during the refractory period.[12] Raising tadpoles the presence of aminoglycoside antibiotics, which is often done prophylactically in laboratories and would be expected to alter the microbiome, reduces the percentage of regenerators in a cohort,[12] suggesting that the microbiome may be important in refractory period regeneration efficiency. Microbiomes are important for wound healing in a lot of model animal systems, including planarians[13,14] and mice.[15,16] Among these examples, indole (an aromatic amino acid metabolite produced by gut bacteria)[14] and the inflammatory cytokine IL-1β[16] have been implicated as critical components of the signalling pathway leading to regeneration.

In *Xenopus*, regeneration in antibiotic-treated tadpoles can be returned to baseline levels by exposing the cut tail surface to heat-killed Gram-negative bacteria or purified lipopolysaccharides (LPS).[12] We hypothesised that TLR4, a Toll-like receptor of the innate immune system that recognises LPS,[17] is exposed to skin bacterial LPS of tadpoles only when the tail is cut.[12] LPS binding of TLR4 on either tissue-resident mesenchymal stem cells[18] or macrophages[17] could produce an inflammatory cytokine response, generating a pro-regenerative environment.

Under laboratory conditions, the most likely source of LPS that could influence tail regeneration is from commensal Gram-negative bacteria on the tadpole skin. Here, we have used 16S ribosomal RNA amplicon sequencing to compare tail skin microbiome composition and regeneration success in three sibships (sibling cohorts) of tadpoles, raised with and without antibiotic gentamicin to disrupt Gram-negative bacterial flora. We also tested the hypothesis that LPS binding to TLR4 elicits a regeneration response, using both an antagonistic LPS purified from *Rhodobacter sphaeroides* (recently renamed as *Cereibacter sphaeroides*[19]), and gene editing of *Tlr4.S*.

2 | METHODS

2.1 | Animal ethics

Procedures for the production of *X. laevis* eggs and embryos were approved by the University of Otago's Animal Ethics Committee as AUP19-01.

2.2 | Animal husbandry

Adult *X. laevis* used in this study are housed within a recirculating aquarium system within PC2 facilities at the University of Otago. The system is supplied with carbon-filtered mains water and frogs are fed twice weekly with salmon pellets. The colony was established in 2004 and has been closed, with no contact with outside animals, since then. Current adults are F_1 or F_2 captive bred.

2.3 | Egg collection and fertilisation

All eggs and embryos used in this work were produced by inducing egg laying in adult female *X. laevis*, weighing 50-100 g, by injecting 500 U of HCG (Chorulon) per 75 g of bodyweight into the dorsal lymph sac. Adult males were killed by immersion in a lethal dose of benzocaine. Eggs were laid into 1× MMR (Marc's modified ringers, pH 7.4: 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4.7H_2O$, 2 mM $CaCl_2$, 5 mM HEPES, 0.1 mM EDTA at pH 8.0) and fertilised in vitro using 50 μl of fresh male *X. laevis* testes, prepared by lightly disrupting tissue using a plastic pestle to release sperm into 1 ml of MMR. Embryos and tadpoles were raised at 18°C in an incubator.

2.4 | Tail regeneration assays

For both the antibiotic treatment and CRISPR/Cas9 editing experiments, groups of tadpoles were raised in 10 mm Petri dishes containing 30 ml 0.1× MMR. For the treatment experiment, tadpoles were raised with or without 50 μg/ml added gentamicin according to treatment group. CRISPR/Cas9 edited tadpoles were raised without gentamicin. Gentamicin was kept constant by adding fresh medium to applicable dishes every 2nd day and was discontinued 1 day post amputation, by which time wound healing is complete. Tail regeneration assays were done at stage 46,[1] in the refractory period[2] before the commencement of feeding. Tadpoles were immobilised using 1/4000 w/v MS222 (tricaine, Sigma) in 0.1× MMR and the distal third of the tail was removed using a sterile scalpel blade. Tadpoles were rinsed in 0.1× MMR to remove MS222. For treatment experiments, tadpole groups were placed back into Petri dishes containing their respective media. For gene-editing experiments, individual tadpoles were placed into 24-well culture plates in 1 ml 0.1× MMR and tail tips were kept for genotyping. Tadpoles were not fed. Tails were scored for regeneration after 7 days as one of four categories: FR (full regeneration, no visible defect, scores 10/10); PG (partial good, tail regenerated but may have a missing fin on one side or a bend in the tail scores 6.6/10); PB (partial bad, at least one core tissue missing, short, often bent or grows along the ventral fin cut site, scores 3.3) or NR (no regeneration, full-thickness epidermis forms over wound site, scores 0). This is based on the method devised by Adams et al.[20] Scoring using these criteria was done on an unblinded basis by a single person across each experiment, and examples are shown in Figure S1.

To assess the ability of LPS to "rescue" regeneration in gentamicin-raised tadpoles, 50 μg/ml or higher of 200× LPS stock was added to tadpole media after tail amputation and rinsing. Tadpoles were incubated in the LPS solution for 1 h before being returned to fresh 0.1× MMR. TLR4 antagonist LPS from *R. sphaeroides* was added for 1 h post amputation in tadpoles raised with no antibiotics.

2.5 | Microbial sampling

Tadpole tail samples (stage 46) were acquired by collecting freshly cut tail tips (posterior third of the anatomical tail) from regeneration assays into 0.2 μl 8-strip PCR tubes, adding 50 μl of filter-sterilised sodium chloride/Tween solution (0.15 M NaCl, 0.1% Tween20), and vortexing for 1 min before storing at −20°C. Negative controls were generated using the same technique but without adding a tail tip. Ninety-six tadpoles (48 gentamicin-raised and 48 untreated) were collected from each of three sibships. The tadpoles were arrayed in 24-well plates with 1 ml 0.1× MMR, incubated at 22°C and assayed for regeneration after 7 days. Tail regeneration was scored as described above, except that the PG and PB regenerates were both classified as "Partial".

2.6 | Tadpole microbial culture assay

A qualitative assay was devised to demonstrate the effect of raising tadpoles in gentamicin on the number of viable bacteria on stage 47 tadpole skin. Individual tadpoles from a single sibship (raised with or without gentamicin) were first washed twice in sterile 0.1× MMR and then vortexed for 20 s in 100 μl of sodium chloride/Tween solution. Fifty microlitres of the resulting solution was added to 1 ml of Luria Broth (LB), diluted 10 fold in LB and spread onto replicate LB agar plates. Plates were incubated at 18°C for 66 h and photographed on a black background.

2.7 | Bacterial culturing

Escherichia coli DH10B strain was grown from glycerol stocks at 37°C in LB overnight with shaking. Commensal bacteria (*Chryseobacterium spp*.) were cultured from adult female *X. laevis* using gentle swabbing of dorsal, ventral and limb skin for a total of 15 s with sterile cotton-tipped swabs (Puritan). Swabs were plated onto Oxoid nutrient agar and incubated at 30°C for 48 h. Colonies were purified by streaking. Two additional bacterial strains were obtained from culture collections in order to characterise the effects of their LPS: *Delftia Wen* et al *1999* (ICMP 19763) was obtained from Manaaki Whenua – Landcare Research NZ[21] and *Rhodobacter sphaeroides* (DSM-158, recently reclassified as *Cereibacter sphaeroides*[19]) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures). Both were grown on Oxoid nutrient agar and incubated at 30°C.

The identity of the commensal *Chryseobacterium spp*. isolate was determined by whole genome sequencing and ANI analysis, using the same methods described by Hudson et al.[22] The isolate was most closely related to *Chryseobacterium sp. MYb7* (ANI 96.7%) and has been deposited in the Manaaki Whenua – Landcare Research culture collection as *Chryseobacterium* XDS4 (ICMP 24359). It is hereafter referred to as *Chryseobacterium spp. XDS4*.

2.8 | LPS extraction from Gram-negative cultures

Purified bacterial isolates were cultured in Oxoid nutrient broth, grown overnight at 30°C, heat killed at 60°C for 60 min and pelleted by centrifugation at 5000×*g* for 10 min. Pellets were resuspended in 10 ml PBS pH 7.2, re-spun and re-suspended and pelleted a final time. Pellets were then frozen at −80°C for at least 2 h before freeze drying in a VaO_2 vacuum chamber at −80°C overnight. LPS was extracted from heat-killed and lyophilised bacteria as described by Yi and Hackett,[23] using TRI-reagent (Sigma). Briefly, each batch used 10 mg lyophilised bacteria and 200 μl TRI reagent in 1.5 ml Eppendorf tubes. LPS was extracted into the aqueous phase with chloroform, and the organic phase was washed 3× to maximise yield. Nucleotides were removed by 10 U DNAse and 20 μg RNaseA treatment for 10 min at 37°C, followed by 20 μg Proteinase K to remove protein and inactivate nucleases for a further 10 min. Samples were dried in an

Eppendorf concentrator plus SpeedVac overnight. Finally, LPS pellets were resuspended in cold 500 IL 0.375 M MgCl in 95% ethanol according to Darveau and Hancock,[24] precipitated at −30°C for 30 min, repelleted at 12 000×*g* for 15 min at 4°C, dried briefly, resuspended in 200 μl ultrapure water and stored as aliquots at −30°C. The estimated concentration of 10 mg/ml was based on a 20% yield of LPS from lyophilised bacteria.[23] LPS was checked by acrylamide gel electrophoresis using a BioRad mini Protean and silver staining (Pierce) according to Laemmli.[25] Duplicate gels were stained with 0.5% Coomassie brilliant blue R250 (Sigma) to confirm no protein. The size was approximated using a 5-μl Novex sharp protein marker.

2.9 | DNA extraction, 16S rRNA amplicon sequencing and analysis of tail samples

DNA from tadpole tails was extracted using a DNeasy PowerLyser PowerSoil DNA extraction kit (Qiagen) according to the manufacturer's instructions, eluted into a final volume of 30 μl and stored at −80°C. Amplification and sequencing of the V4 hypervariable region of 16S rRNA gene by Illumina MiSeq were performed for as described previously by Caporaso et al. (2011) using primers 515F/862R.[26] Sequencing of 229 samples was done at Argonne National Laboratory, Illinois, USA, and used peptide nucleic acid (PNA) PCR clamps to inhibit the amplification of host mitochondrial sequences.[27] Amplicon sequences (2 × 250 bp) were processed using the DADA2 package (version 1.6.0) in R[28] according to authors' recommended best practices. The taxonomy was annotated using the naïve Bayesian classifier method with the Silva reference database version 128.[29] Downstream analyses were performed using R (version 3.4.3), packages vegan (version 2.4.6)[30] and phyloseq (version 1.22.3).[31] Samples with fewer than 1500 reads were excluded from further analysis. Sequence data for all samples have been deposited with NCBI (BioProject ID PRJNA780297).

2.10 | CRISPR/Cas9 targeting of *Tlr4.S*

ChopChop v2[32] was used to identify four unique sgRNA sequences from *X. laevis Tlr4.S* (Table 1). EnGen sgRNA oligo designer v1.12.1 tool (NEB) was used to generate 55 bp oligos. These were synthesised by IDT and converted into sgRNAs using the EnGen Cas9 sgRNA kit (NEB) according to instructions. sgRNA was extracted using phenol/chloroform and precipitated with ammonium acetate and ethanol, resuspended in 30 μl of ultrapure water (Sigma) and stored at −80 °C in 2 μl aliquots. Typically, this method produces concentrations of around 500 ng/μl; exact concentrations for each sgRNA are provided in Table 1. Working dilutions of sgRNA were made just prior to injection by diluting 3 or 5 fold. EnGen *S. pyogenes* Cas9 NLS (NEB) protein (0.3 μl) was loaded with sgRNA (1 μl for 1:3, 0.6 μl for 1:5) by incubating them together with ultrapure water for 5 min at 37°C in a total volume of 3 μl. Freshly fertilised *X. laevis* eggs were de-jellied in 2% cysteine pH 7.9 and rinsed three times with 1× MMR. Embryos were selected for injection based on the appearance of sperm entry points and placed into a well cut into a 2% agar lined Petri dish containing 6% Ficoll 400 in 1× MMR. Cas9/sgRNA solution was loaded by backfilling into a glass capillary needle (Drummond) pulled to a fine point using a Sutter P92 needle puller and the end clipped with fine forceps. The needle was loaded onto a Drummond Nanoject II micropipette held with a MM3 micromanipulator, and embryos were injected with 9.2 nl of Cas9/sgRNA. Fifty embryos were injected at each dilution, and 50 controls were injected with only Cas9 protein. After 2–3 h, embryos were placed in 3% Ficoll, 0.1× MMR. After 18 h, they were moved to 0.1× MMR.

2.11 | Genotyping and editing analysis

For cohort genotyping, eight randomly chosen single embryos at stage 11-12 were collected into 0.2 μl PCR tubes and any liquid was replaced with 150 μl of 5% Chelex beads in TE (Tris/EDTA buffer, pH 8.0) with 30 μg Proteinase K. Following this, they were homogenised briefly by pipetting and incubated at 56°C for 4 h, then at 95°C for 5 min to inactivate the enzyme. Chelex extracts were used directly for PCR and stored at 4°C. For confirming editing in tail tips, the same process was followed except that 56°C incubations were overnight, and vortexing was used instead of pipetting to disrupt the tissue.

PCR primers (Table 2) were as suggested by Chopchop v2[32] for each sgRNA, amplifying approximately 250 bp around the target site. One microlitre of Chelex extracted DNA was amplified with the appropriate primers and MyTaq polymerase (Bioline) in a 20 μl volume. A T7 endonuclease I assay was used to initially confirm editing. PCR amplicons were cleaned using ExoSap-IT (Applied Biosystems) and sent for Sanger sequencing (Genetic Analysis Service, University

TABLE 1 sgRNA for *Tlr4.S*, ranked by ChopChop v2, with PAM in bold

sgRNA	Sequence	Concentration (ng/μl)	Editing efficiency %	Frameshift %
Rank 1	CCGGTAACCCAATACGCCAT**TGG**	656.0	51.4	77.3
Rank 2	TAGAGTACCTTGATCTCACC**AGG**	563.6	59.3	78.0
Rank 15	GATGAGATTGTAGGAGATCC**AGG**	528.4	50.3	48.4
Rank 23	TGTGGATCCCAATGGCGTAT**TGG**	596.4	48.7	80.0

Notes: Stock concentrations for each sgRNA are provided, as well as predicted efficiency of editing and frameshift from InDelphi.[59]

sgRNA	Forward primer	Reverse primer	Product size (bp)
Rank 1	TGAGGATCTAGCATTTTCAGGC	TGTCGTGAGATGCAGAGATTTT	229
Rank 2	AGCTTCAACCCCCTTAGACATA	CATGGGCCTTATTTGAGTGATG	228
Rank 15	ATTCCTGAAGGGACTTTTTCGT	GAACAGTCAAAAGGGTTTCCTG	221
Rank 23	AAATTGTGTTTCTCTGCAGGTG	TGTCGTGAGATGCAGAGATTTT	266

TABLE 2 Genotyping primers for *Tlr4.S* Crispants

of Otago) using the primer predicted to be furthest from the editing site. TIDE v2 (Tracking of Indels by Decomposition)[33] and DECODR (Deconvolution of Complex DNA repair)[34] were used to assess the editing from the sequence trace files. An example of the editing by sgRNA rank 15 is shown in Figure S2.

2.12 | Statistical analyses

Graphs were made using Graphpad Prism v9.01 or R v4.1.0 (ggplot2[35]). Corresponding analyses of significant differences were performed in the same packages. Unpaired *t*-tests or one-way ANOVA with Tukey's post hoc test was used to compare the percentage of tadpoles in each dish that attempted regeneration, between untreated, antibiotic-raised and/or LPS-treated groups. Regeneration quality scores comprised of categorical data (FR, PG, PB and NR) were compared using Extended Cochran–Armitage tests or Linear × Linear association tests, followed by post hoc pairwise ordinal independence test with Benjamini–Hochberg correction for multiple testing (P. adjust). The level of CRISPR editing between regeneration categories was compared using unpaired t-test or Wilcoxon Rank Sum following Shapiro–Wilk test of normality. Statistical analyses and raw data can be found in the Supporting information S1.

Relative abundance plots were created in R v4.1.0 using the ggplot2 v3.3.5[35] and microshades v0.0.0.9000[36] packages. For beta diversity analysis and visualisation, Bray–Curtis distance was calculated between samples after glomming data to genus level and normalising to relative abundance, and the vegan package[30] was used for permutation-based ANOVA. Bacterial genera that were associated with regeneration after accounting for gentamicin use were determined by using EdgeR[37] to fit a quasi-likelihood negative binomial generalised log-linear model with Benjamini–Hochberg false discovery correction $q < 0.01$. Only genera seen at least 14 times in at least 20% of samples were analysed with EdgeR. The R code used for all 16S rRNA data processing and analysis is supplied at https://gitlab.com/morganx/xenopus1.

3 | RESULTS

3.1 | The microbiome of tadpole tail skin is consistent within, but variable between sibships, and is altered dramatically by raising tadpoles in antibiotics

Embryos were collected from three sibships and raised from the 4-cell stage in 0.1× MMR with or without 50 μg/ml gentamicin (Figure 1A). At stage 46, 48 tadpoles from each cohort were subjected to partial tail amputation, with the tail tips collected for 16S ribosomal RNA sequencing. Regeneration was scored after 7 days. Raising embryos and tadpoles in gentamicin significantly reduced the number of tadpoles that regenerated their tails for all three sibships (Figure 1B) and also significantly decreased the quality of regeneration (Figure 1B′). Sibship accounted for 43% of microbial community variation within tails ($R^2 = 0.43$, $p < .001$, PERMANOVA), while gentamicin use accounted for 14% of variation ($R^2 = 0.14$, $p < .001$, PERMANOVA) (Figure 1C). Gentamicin is a broad-spectrum aminoglycoside antibiotic that targets Gram-negative bacteria primarily, but not exclusively.[38] Consistent with this, the bacterial population of untreated tadpole tails comprised almost entirely Gram-negative taxa, while Gram-positive taxa were much more abundant in gentamicin-treated tails (Figure 1D). Bacterial composition was largely consistent within sibships, but variable between sibships (Figure 1E). Sibships A and B were dominated by alphaproteobacteria, while betaproteobacteria were more abundant in Sibship C. Both alpha- and betaproteobacteria were less abundant in the gentamicin-treated groups (Figure 1E).

We next examined how specific bacterial genera were affected by gentamicin treatment (Figure 2A) and asked if any of these were associated with successful regeneration (Figure 2B). Without treatment, each sibship was dominated by a single genus—either *Shinella* (Sibships A and B) or *Delftia* (Sibship C). Raising tadpoles in gentamicin reduced the dominance of the primary colonising genus, allowing the detection and/or growth of, less- abundant taxa (Figure 2A). EdgeR[37] identified six bacterial genera that were present on at least 20% of tail samples and were associated with successful regeneration (Figure 2B). These six genera varied in their relative contribution to the untreated microbial community and were generally proportionately reduced by gentamicin treatment.

One possible explanation for the relative increase in Gram-positive taxa detected on the skin of tadpole tails when animals are raised in gentamicin is that an overall reduction of commensal bacteria allows Gram positives to bloom. To test this hypothesis, tadpoles from two further sibships were raised to stage 47 with or without gentamicin. Bacteria were recovered from the exterior surface of each tadpole and plated onto LB agar (Figure 3A). Plates inoculated from treated tadpoles generated few or no colonies, while plates from untreated tadpoles generated large numbers of colonies (Figure 3B), indicating that gentamicin was indeed effective in reducing the number of viable bacteria on tadpole skin. Control plates with no tadpole material failed to produce discernible colonies (Figure 3C). The results of these cultures suggest that overall bacterial load is reduced on the skin of these tadpoles, and the observed reduction in total number of 16S rRNA reads within normalised sequencing libraries that were generated

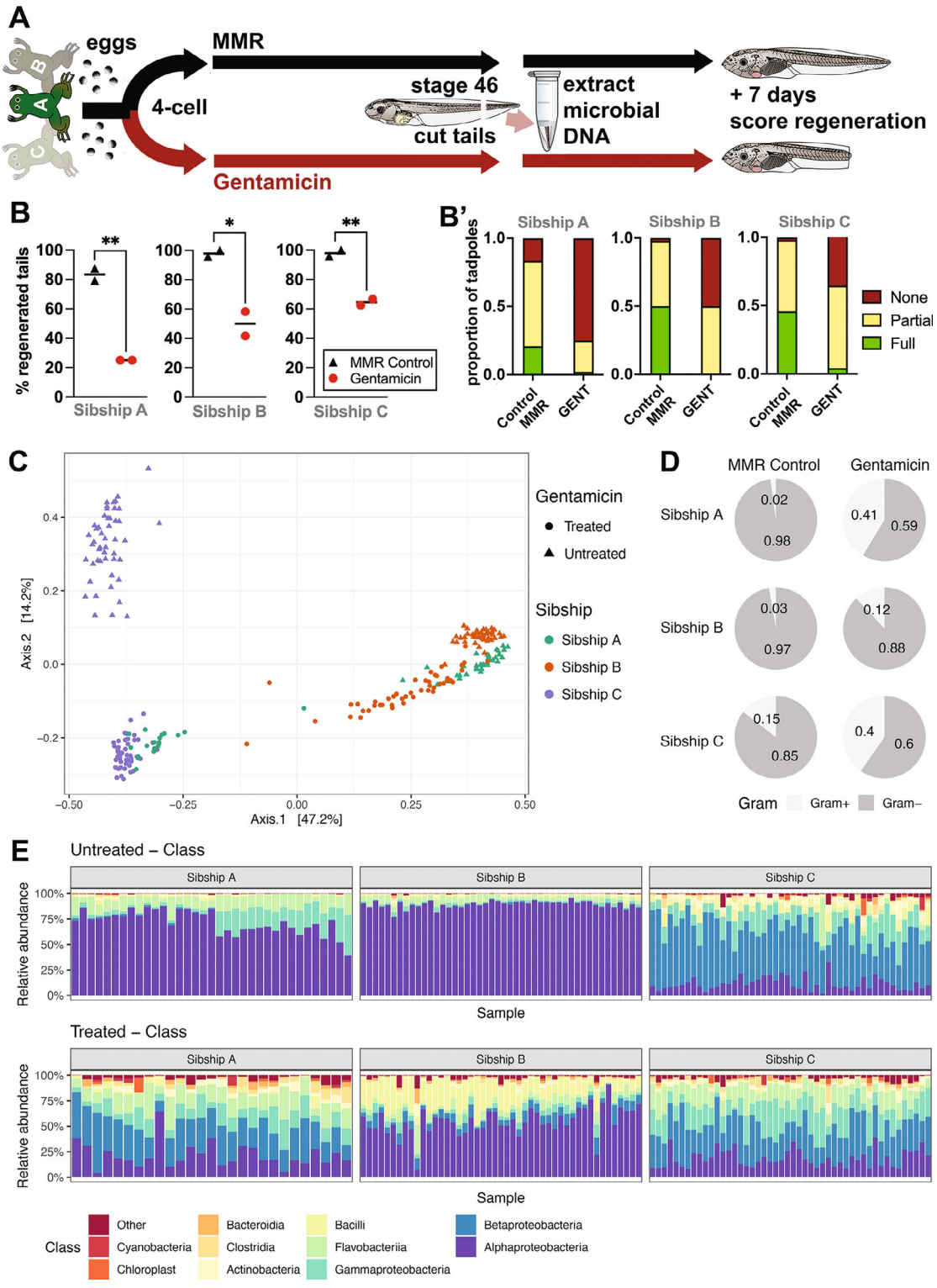

FIGURE 1 The tadpole tail skin microbiome varies between sibships and can be altered dramatically by raising tadpoles in antibiotics. (A) Schematic of the experimental design. Three sibships of 4-cell embryos were randomly assigned to gentamicin-treated and control groups. Tail samples for microbiome analysis were obtained at stage 46 from two replicate cohorts of 24 tadpoles for each treatment and sibship. Tadpoles were scored for regeneration 7 days after tail amputation. (B) Regeneration data from three tadpole sibships. Each point represents the percentage of tadpoles regenerating any tissue at all, is the sum of full, partial good and partial bad tadpoles and is a replicate Petri dish with sample size of 24 tadpoles per dish, with the exception of controls for Sibship B where $N = 22$ as two died in each before they could be scored for regeneration. Unpaired *t*-tests, $^{*}p < .05$, $^{**}p < .01$. (B′) Stacked categorical graphs comparing regeneration phenotypes for each sibship. Linear-by-Linear association test, $^{****}p < .0001$. (C) Principal coordinates analysis (PCoA) ordination plot of tadpole tail samples with >1500 reads, calculated based on Bray–Curtis distance. (D) Pie charts showing the percentage of Gram-negative versus Gram-positive annotated reads for each sibship when raised with or without gentamicin. (E) Relative abundance of the 10 most abundant bacterial classes in tadpole tail skin, stratified by sibship and treatment status. Raw data can be found in Supporting information S1

from gentamicin-treated tadpole tail samples is consistent with this (Figure S3).

3.2 | LPS from commensal *Chryseobacterium spp. XDS4* or from a *Delftia spp*. isolate can rescue regeneration in gentamicin-raised tadpoles

Our previous work showed that the addition of commercially purified *E. coli* or *Pseudomonas aeruginosa* LPS to the tadpole media immediately after tail amputation rescues regeneration of antibiotic-raised tadpoles to untreated levels.[12] We hypothesised that LPS from the commensal genera that we had identified as overrepresented in regenerating tadpoles would also promote regeneration of refractory stage tadpoles. We adapted a method for extracting LPS from cultured bacteria and benchmarked this against commercial preparations of *E. coli* 055:B5 LPS. Both commercial and lab-extracted *E. coli* LPS were added to gentamicin-treated tadpoles, in an attempt to rescue their regeneration ability (Figure S4A). Tadpoles from two sibships raised in gentamicin showed a significantly reduced ability to regenerate compared to untreated controls (Figure S4B,C). When added back to treated tadpoles, both forms of *E. coli* LPS were able to rescue the frequency of tadpoles undergoing tail regeneration to control levels (Figure S4B,C). The quality of the regenerates was fully rescued in one of the sibships (Figure S4C′) but only partially in the other (Figure S4B′).

We next attempted to isolate regeneration-associated commensal species directly from adult female *X. laevis* skin swabs. We

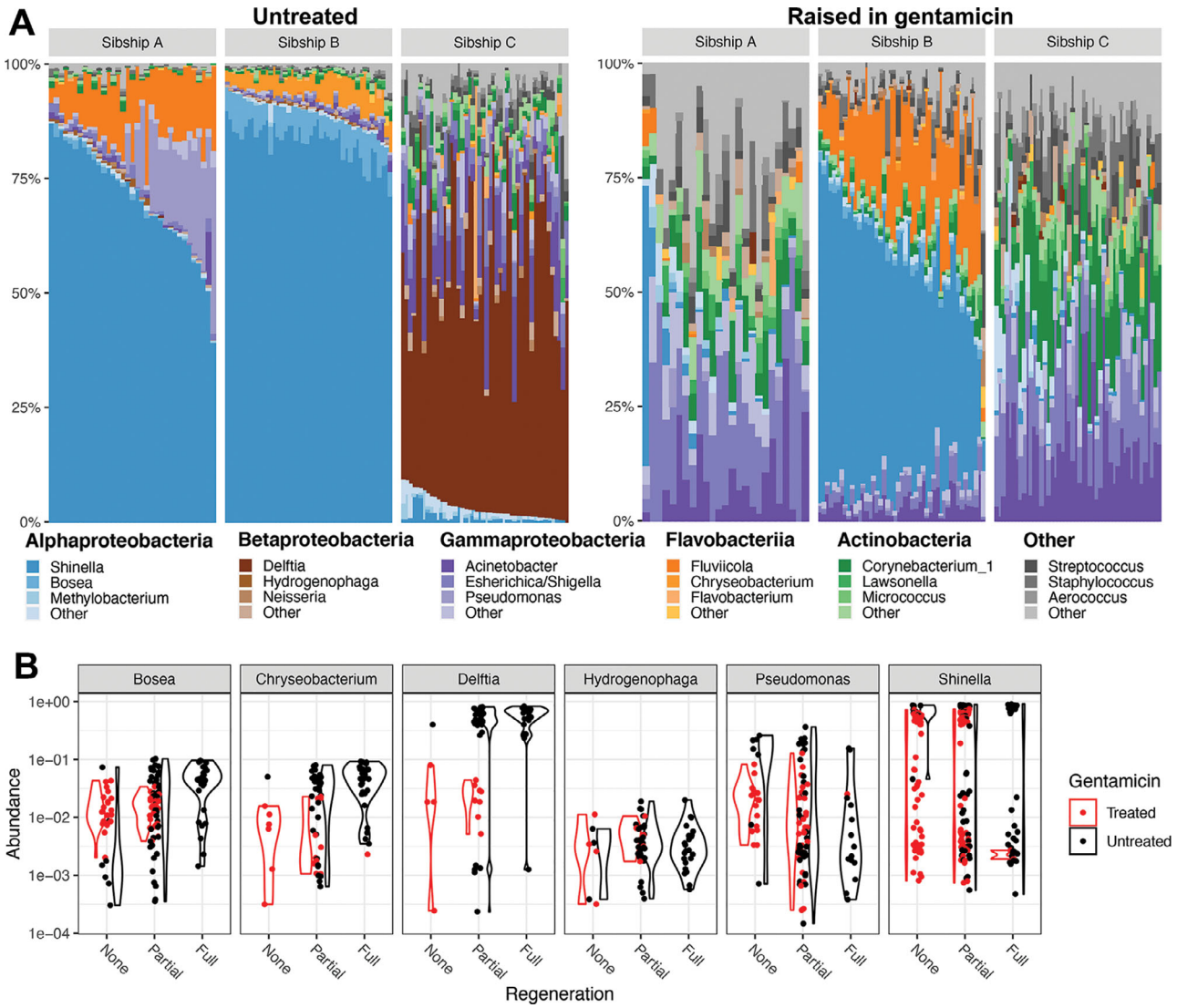

FIGURE 2 Genus-level interactions between sibship, antibiotic treatment and regeneration. (A) The relative abundance of genera within the five most abundant bacterial classes in treated and untreated sibships, highlighting the three most abundant genera in each. Read counts were rarefied to 1500 reads. (B) Violin plots show log-transformed relative abundance (*y*-axis) of six genera positively associated with regeneration ($q < 0.01$, Benjamini–Hochberg false discovery correction) stratified by gentamicin status (colour). Raw data can be found in the Supporting information S1

successfully isolated two of the genera identified as regeneration biased by the differential abundance analysis (Figure 2B), a novel *Shinella*[22] and a *Chryseobacterium spp*. LPS was extracted from *Chryseobacterium spp. XDS4*, as *Chryseobacterium spp*., was the most overrepresented in successfully regenerating tadpoles after *Delftia spp*. (Figure 2B). The ability of *Chryseobacterium* LPS to rescue regeneration was compared to 50 μg/ml of *E. coli* 055:B5 LPS (Figure 4). In all three sibships tested, LPS from *Chryseobacterium spp. XDS4* was at least as effective as *E. coli* LPS in its ability to rescue tail regeneration following gentamicin treatment. A 50 μg/ml dose was able to restore regeneration to levels comparable with those seen in control (MMR) tadpoles and increased doses did not result in improvement of the regeneration outcome (Figure 4B-D). For each sibship, we were able to rescue regeneration in antibiotic-raised tadpoles to the level seen in control tadpoles, which varied with sibship (86%, 100% and 89% for Figure 4B-D, respectively). The quality of regeneration was not able to be fully rescued to control levels by LPS in one of the three sibships (Figure 4B′), but full rescues were achieved by 250 μg/ml of *Chryseobacterium* LPS (Figure 4C′) or by 50 μg/ml (Figure 4D′).

Delftia was abundant in Sibship C (Figure 2A), but we did not culture any *Delftia spp*. from frog skin. As *Delftia* was the most overrepresented genus in regenerating tadpoles (Figure 2B), LPS was prepared from an isolate of *Delftia* (ICMP19763) obtained from Manaaki Whenua – Landcare Research New Zealand. This LPS was found to be at least as effective as *Chryseobacterium spp. XDS4* and *E. coli* LPS at rescuing regeneration in gentamicin-raised tadpoles (Figure 5). Tadpoles from all three sibships reached control regeneration levels when raised in gentamicin and treated with *Delftia* LPS immediately post amputation (Figure 5A-C). Regeneration quality was also fully rescued by 50 μg/ml *Delftia* LPS in all three sibships (Figure 5A′-C′).

3.3 | Addition of antagonistic LPS from *Rhodobacter sphaeroides* or CRISPR/Cas9 editing of TLR4 reduced regeneration quality in untreated tadpoles

We had previously suggested that TLR4 might act as the receptor for LPS,[12] because TLR4 is the most specific PAMP (Pathogen-Associated Molecular Pattern) for LPS and is known to activate the transcription factor NF-κB.[17] To directly test the role of TLR4 in the regeneration pathway, penta-acetylated LPS from *R. sphaeroides*, a TLR4

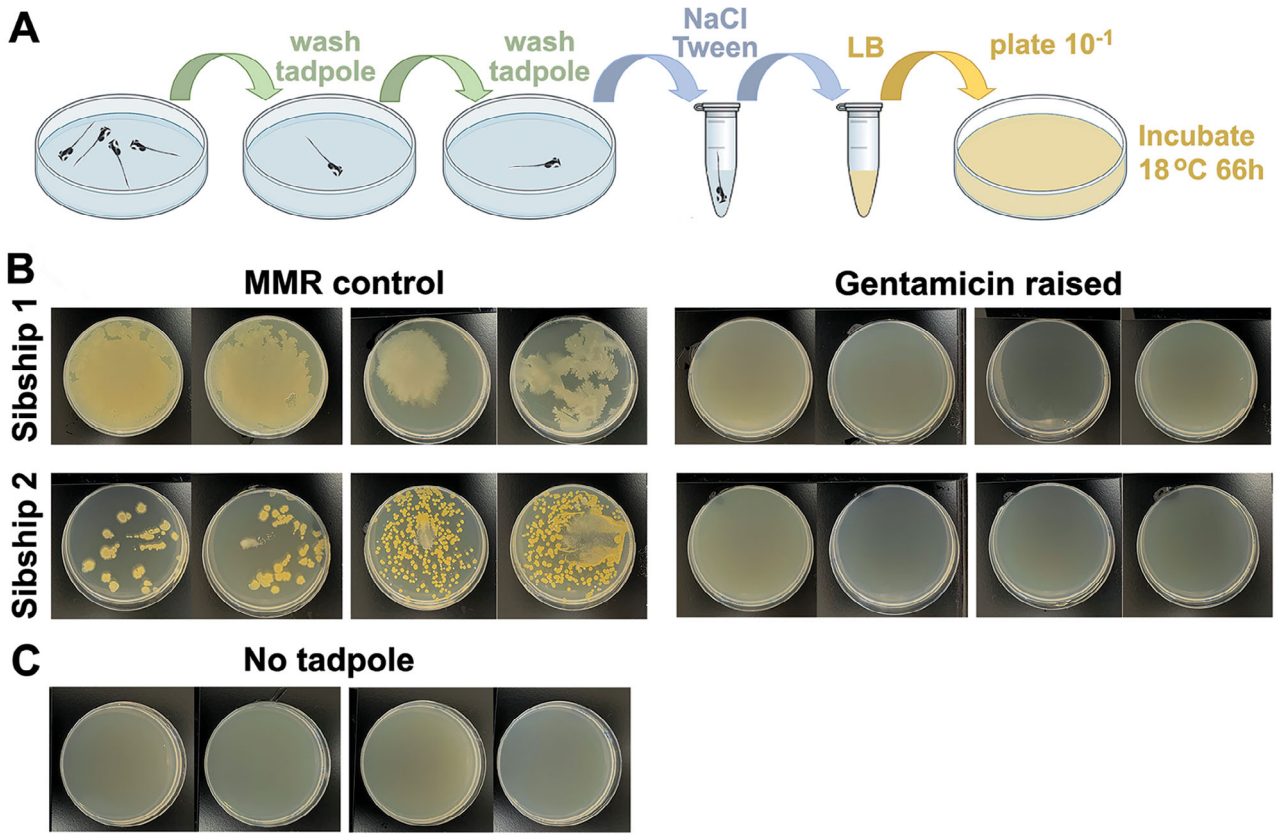

FIGURE 3 Raising tadpoles in 50 μg/ml gentamicin dramatically reduces the number of viable bacteria grown from tadpole skin. (A) Schematic of the method used to capture bacteria from single stage 47 tadpoles. After being raised in either MMR or gentamicin solution, a selected tadpole was washed twice in MMR and vortexed for 20 s in 100 μl NaCl/Tween20. Fifty microlitres of the solution was then added to 1 ml Luria Broth and two replicate plates spread. (B) Plates photographed after 66 h at 18°C. Two tadpoles from each sibship, raised ± gentamicin are shown. (C) Controls prepared as above but with no tadpole, to ensure no contamination from the environment

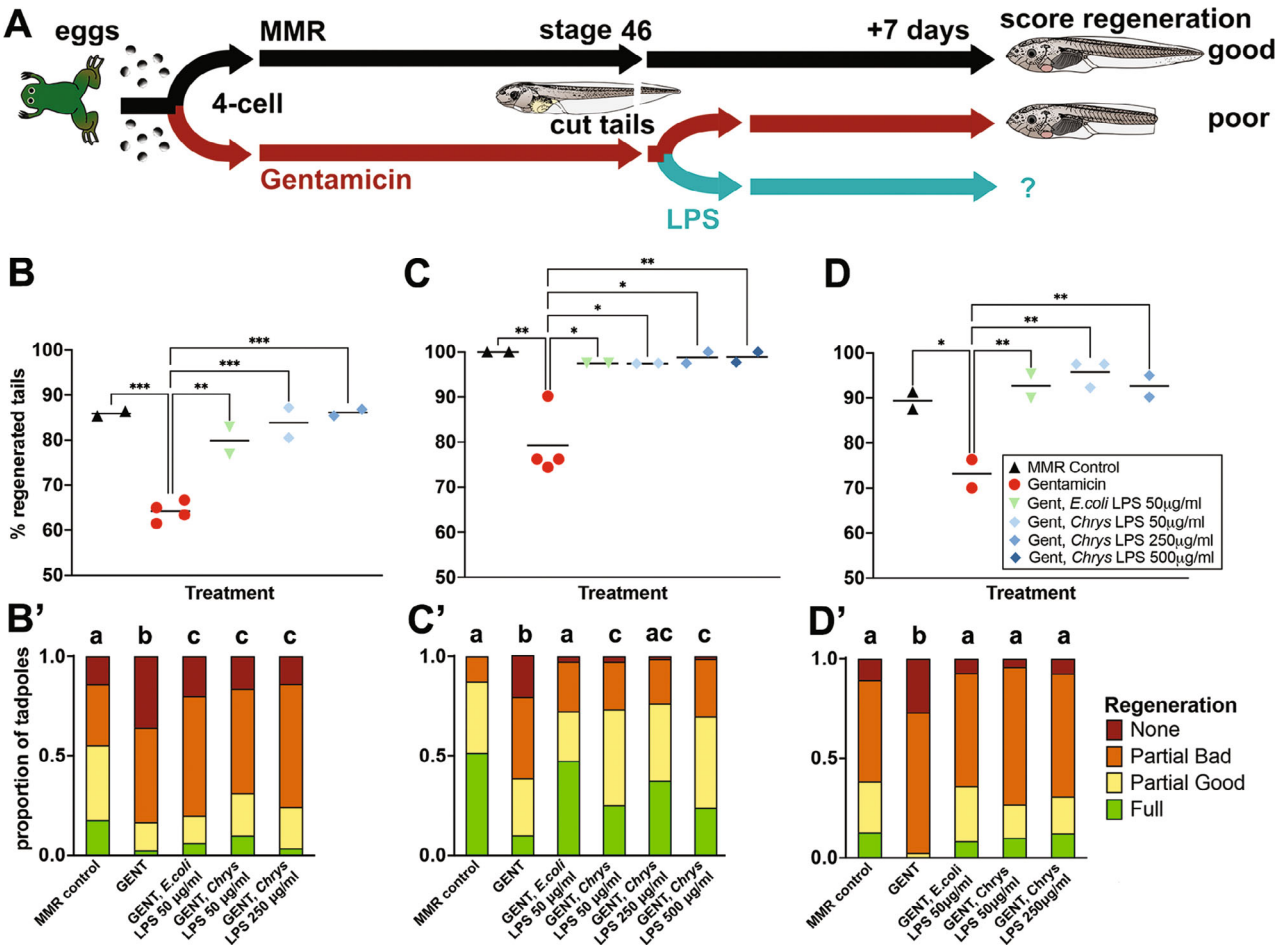

FIGURE 4 LPS from the commensal bacterium *Chryseobacterium spp. XDS4* rescues regeneration in stage 46 tadpoles raised in the antibiotic gentamicin (gent). (A) Timeline of treatments. (B–D) Data from three sibships of tadpoles. Each point represents the percentage of tadpoles regenerating any tissue at all, is the sum of full, partial good and partial bad tadpoles and is a replicate Petri dish with sample size of 38-48 (A), 35-43 (B) or 23-43 tadpoles per dish. (C) One-way ANOVA with Tukey post hoc comparisons of all means. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. (A′–C′) are stacked categorical graphs of the same tadpoles, showing the proportion of each phenotype by dish. Compact letter display has been used to indicate statistical significance; each treatment is assigned a letter, with treatments within the same letter group having no statistically significant difference from each other. Extended Cochran–Armitage test, followed by post hoc pairwise ordinal independence test with Benjamini–Hochberg correction. Raw data can be found in the Supporting information S1

antagonist[39,40] was added to tadpoles not exposed to gentamicin (Figure 6A). Tadpoles treated with a commercial preparation of *R. sphaeroides* LPS (Invivogen) at 50 µg/ml did not significantly reduce the percentage of tadpoles undergoing regeneration but did significantly reduce the quality of regeneration compared with untreated controls (Figure. 6B,B′, Asymptotic linear-by-linear association test, $p = .0428$). We also prepared LPS from *R. sphaeroides* ourselves, and this was able to reduce regeneration quality to a level similar to those seen in gentamicin-treated sibling tadpoles (Figure 6C,C′). While the standard dose of 50 µg/ml did not significantly reduce regeneration quality compared to controls, an increased dose of 250 µg/ml was able to achieve outcomes similar to those in gentamicin-treated tadpoles. A further increase to 500 µg/ml did not result in any further reduction in regeneration (Figure 6C,C′).

As a second approach, we used CRISPR/Cas9 to disrupt the *Tlr4.S* gene. *Xenopus laevis* is allotetraploid,[41] but there is only a single copy of TLR4. We predicted that editing would lead to gene function disruption and a subsequent reduction of regeneration in crispants. Four sgRNAs were designed and trialled to determine their efficiency in editing *Tlr4.S* (Figure 7A, Tables 1 and 2). Of these, sgRNA rank 15 at a 1:3 concentration, predicted to cause a frameshift resulting in a premature stop codon (Figure 7A), was the only sgRNA to achieve a high level of editing in embryos (74%, Figure 7B, Figure S2). The effect of *Tlr4.S* editing on frequency of tadpole tail regeneration was not significant, but the quality of regeneration in crispants was significantly lower than for controls (Extended Cochran–Armitage test, $p = .000000132$). In the same sibship, *R. sphaeroides* LPS also reduced regeneration quality ($p < .0001$). Gentamicin-raised tadpoles had significantly lower levels of regeneration than *R. sphaeroides* treated or crispant tadpoles (Figure 7B). Sequence analysis of embryos using the three other sgRNA, or with Cas9 alone, showed no significant editing, and regeneration quality was indistinguishable from controls

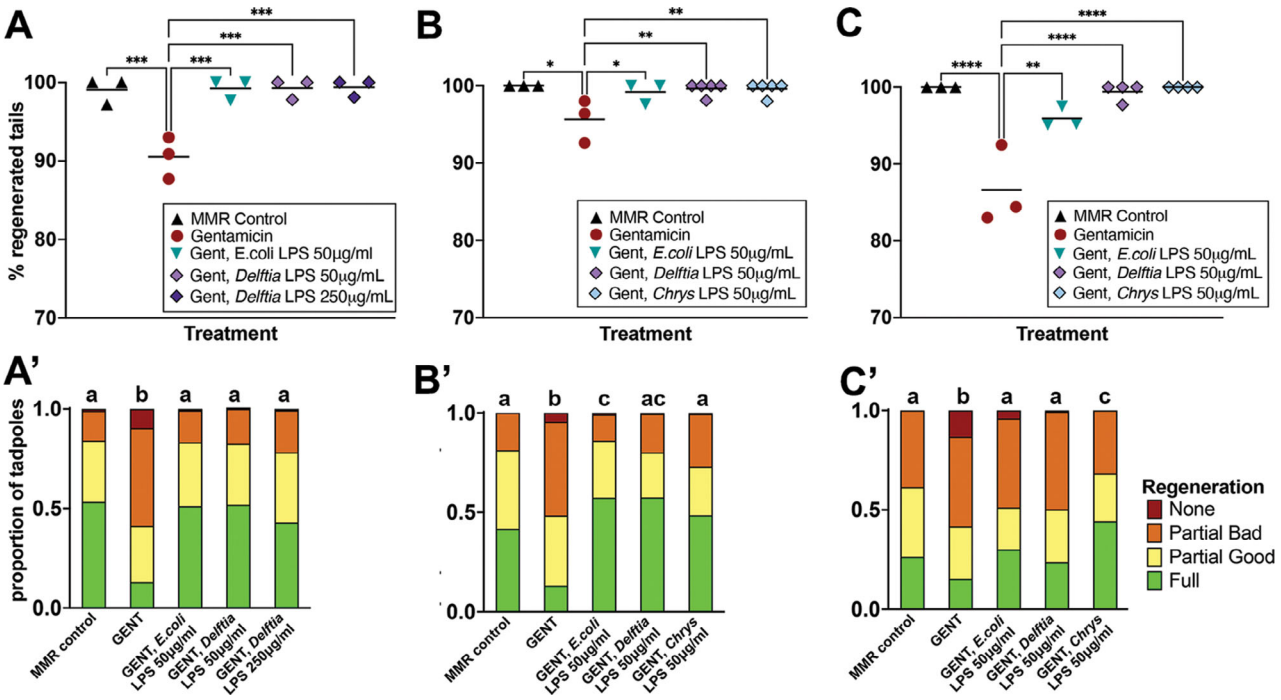

FIGURE 5 LPS from an exogenous *Delftia spp*. rescues regeneration in stage 46 tadpoles raised in the antibiotic gentamicin (gent). Timeline of treatments as for Figure 4A. (A–C) represent data from three sibships of tadpoles. Each point represents the percentage of tadpoles regenerating any tissue at all, is the sum of full, partial good and partial bad tadpoles and is a replicate Petri dish with sample size of 32-65 (A), 23-60 (B) or 40-65 tadpoles per dish (C). One-way ANOVA with Tukey post hoc comparisons of all means. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. (A′–C′) are stacked categorical graphs of the same tadpoles, showing the proportion of each phenotype by dish. Compact letter display has been used to indicate statistical significance; each treatment is assigned a letter, with treatments within the same letter group having no statistically significant difference from each other. Extended Cochran–Armitage test, followed by post hoc pairwise ordinal independence test with Benjamini–Hochberg correction. Raw data can be found in the Supporting information S1

(Figure 7B). Taken together, we suggest that partial inhibition of TLR4 signalling, by either excess antagonist LPS or partial gene editing with CRISPR/Cas9, does reduce the quality of tadpole regeneration.

Individual tadpole tail clips from the sgRNA rank 15 group were also checked for editing, which ranged from 19% to 54% across 27 individuals. The mean editing in the cohort was 34%, with frameshift editing at just 17.8%. To see if individual editing levels influenced the regenerative outcome of tadpoles, we compared editing levels in tadpoles grouped by the four regeneration categories. No significant difference in either total editing or frameshift editing percentage was evident between the groups (Figure 7C) except when frameshift editing in the FR and PG groups was compared. Overall, this indicates that a particular tadpole was not less likely to regenerate if its *Tlr4.S* editing level was higher.

4 | DISCUSSION

Amphibian tadpoles, like all metazoa, support populations of microorganisms that interact with their hosts through various mechanisms. Here, we show that the tadpole skin microbiome is highly variable and can be manipulated by raising embryos in the antibiotic gentamicin. Six Gram-negative genera, including *Delftia* and *Chryseobacterium*, were over-represented in tadpoles that successfully regenerated their tails. Regeneration could be rescued in antibiotic-raised tadpoles by adding LPS from commensal *Chryseobacterium spp. XDS4*, *Delftia Wen* et al *1999* or *E. coli*. Conversely, regeneration was impaired in tadpoles exposed to an antagonistic LPS isolated from *R. sphaeroides*. Disrupting *Tlr4.S* using CRISPR/Cas9 also reduced regeneration quality, but not quantity, at the level of the cohort. However, we found that the editing level of individual tadpoles was not a good predictor of regenerative outcome.

4.1 | The *X. laevis* pre-feeding tadpole skin microbial community varies with sibship, lacks diversity and can be manipulated with antibiotics

Gram-negative bacteria, in particular Proteobacteria, were dominant over Gram-positive phyla in the tadpoles' unmodified microbiome (Figure 1). However, the dominant bacterial clades varied between sibships; the alphaproteobacteria class was predominant in two sibships (A and B), while betaproteobacteria dominated the third (C). Sibship B had highest detected levels of alphaproteobacteria and

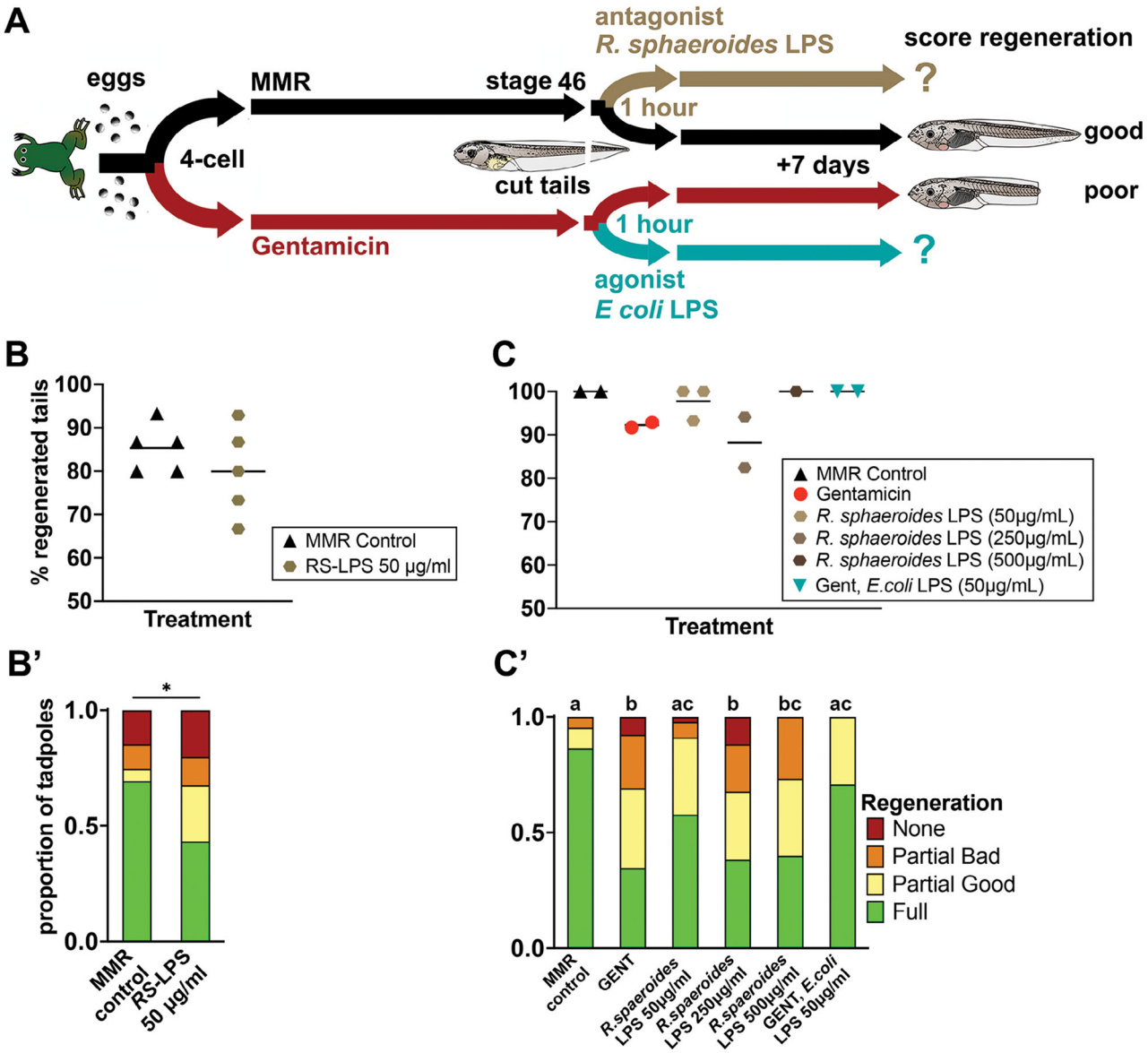

FIGURE 6 *Rhodobacter sphaeroides* LPS, a TLR4 antagonist, can significantly reduce regeneration quality, but not quantity. (A) Timeline of treatments. Exposure of the cut tail stump to agonistic LPS should enhance regeneration in antibiotic-raised tadpoles, as in Figures 2–4, and antagonistic LPS (RS-LPS) is expected to reduce regeneration in naturally raised tadpoles. (B and C) Scatterplots where each point represents the percentage of tadpoles regenerating any tissue at all, is the sum of full, partial good and partial bad tadpoles and is a replicate Petri dish with sample size of $N = 15$ (B) or 11-17 tadpoles per dish (C). (B) 50 µg/ml ultrapure RS-LPS (Invivogen) treatment versus controls. Unpaired *t*-test showed no significant difference between groups. (C) Post-amputation treatment with three concentrations of antagonistic-extracted RS-LPS was compared to control tadpoles and gentamicin-treated tadpoles with or without *E. coli* LPS rescue. One-way ANOVA with Tukey post hoc comparisons of all means showed no significant differences between groups. (B′ and C′) are stacked categorical graphs of the same tadpoles, showing the proportion of each phenotype by dish. Compact letter display has been used to indicate statistical significance; each treatment is assigned a letter, with treatments within the same letter group having no statistically significant difference from each other. Extended Cochran–Armitage test, followed by post hoc pairwise ordinal independence test with Benjamini–Hochberg correction. *$p < .05$. Raw data can be found in the Supporting information S1

retained these at higher levels than in Sibships C or A, when raised in gentamicin. This variation between tadpole cohorts may be partly due to host genetics but is probably also attributable to environmental factors. While *Xenopus* microbiome work is in its infancy, Piccini et al.[42] found that although the adult *X. laevis* skin microbiome is subject to strong selective pressures from the host, tadpole microbiomes were more variable and influenced by environmental conditions. Interestingly, the microbiomes of the older, premetamorphic tadpoles in the Piccini study[42] were also dominated by proteobacteria, although were not dominated by single genera as ours were. However, this is almost certainly affected by differences in stage/age and sample collection methods. Piccini et al. swabbed month-old tadpoles (expected stage

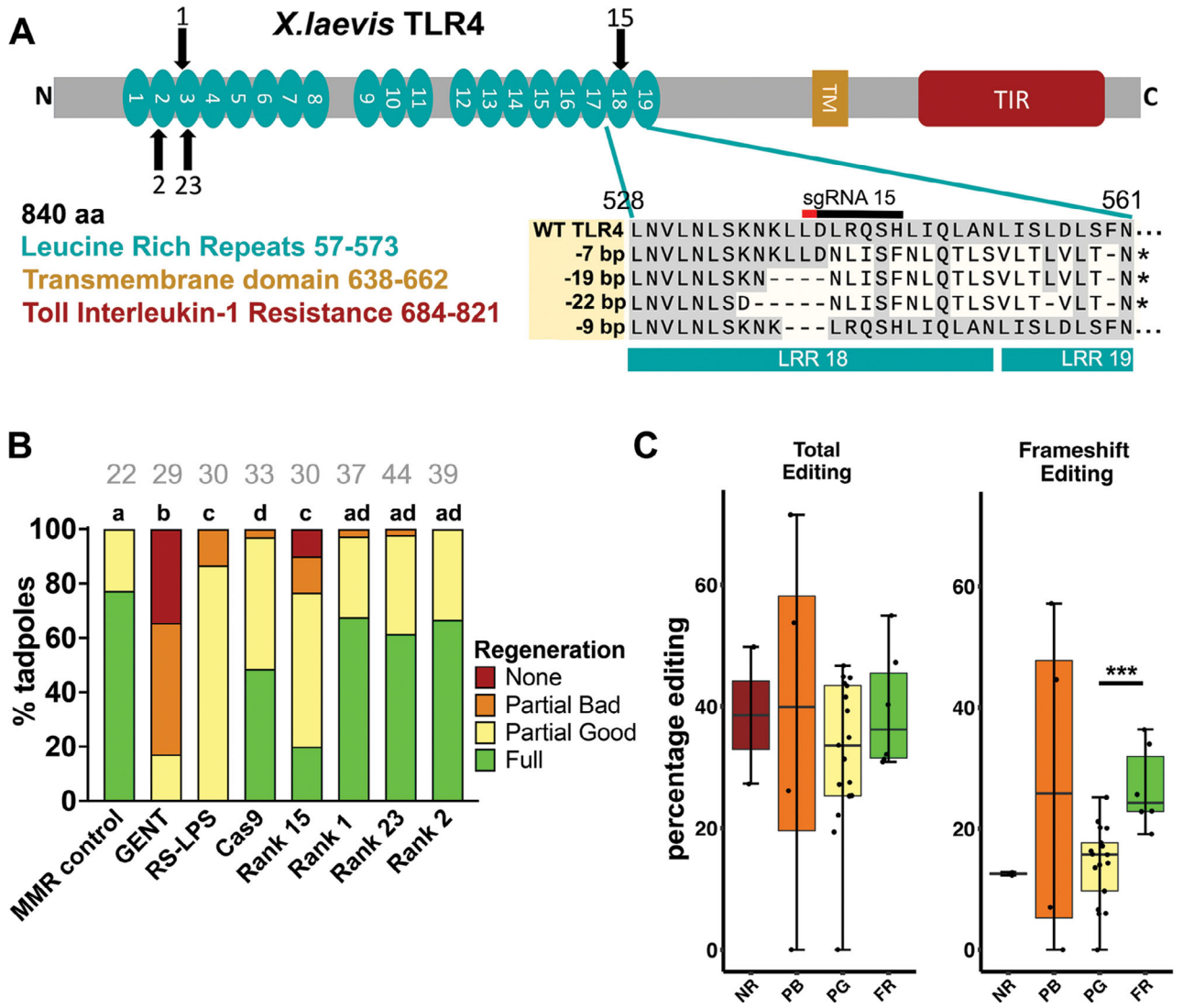

FIGURE 7 TLR4 editing with CRISPR/Cas9 correlates with reduced regeneration score at sibship but not individual tadpole level. (A) Schematic of *X. laevis* TLR4 protein, showing 19 predicted extracellular LRR domains, an internal Toll-interleukin-1 inhibition domain (TIR) predicted by NCBI CDD and a single transmembrane domain (predicted by TMHMM server v2.0).[60] Black arrows show targets of sgRNA, numbers associated with arrows indicate the specific sgRNA. The most common deletions generated by sgRNA rank 15 result in a + 1 frameshift which leads to a stop codon that truncates the protein mid-19th LRR domain. A less commonly occurring −9 bp deletion results in the loss of three amino acids from LRR 18. (B) Stacked categorical graphs of tadpole regeneration, showing the proportion of each phenotype by treatment. MMR controls are unmanipulated embryos, gentamicin is embryos raised in 50 μg/ml gentamicin from 4 cell stage to 1 day post amputation. No other embryos in this set were raised in antibiotics. RS-LPS is *R. sphaeroides* LPS, a natural TLR4 antagonist. Four different sgRNA were used, average editing for eight randomly chosen stage 10 embryos was 74% for sgRNA rank 15 and <10% for the other sgRNA. Tadpoles at stage 46 had the posterior third of the tail removed using a scalpel blade and were scored for regeneration quality a week later. Compact letter display has been used to indicate statistical significance; each treatment is assigned a letter, with treatments within the same letter group having no statistically significant difference from each other. Grey numbers above bars indicate sample size (N). Extended Cochran–Armitage test, followed by post hoc pairwise ordinal independence test with Benjamini–Hochberg correction. (C) Boxplot of total editing percentage (C) and frameshift editing percentage (C′) in tadpole tail clips from each regeneration category. Shapiro–Wilk test of normality, followed by unpaired *t*-test (Editing) and Wilcoxon Rank Sum (Frameshift). FR, full regeneration; NR, no regeneration; PB, partial regeneration (Bad); PG, partial regeneration (Good). ***$p < .001$. Raw data can be found in the Supporting information S1

54-55, length 60-80 mm) that had been fed algae and were housed in aquaria,[42] whereas those in our study were maintained in Petri dishes in MMR at a constant 18°C, were approximately 6-7 days old (stage 46, 9-12 mm length) at sampling and had never been fed. Further, Piccini's tadpoles and frogs were routinely raised for the 1st week in penicillin and streptomycin and could, therefore, have acquired their microbiome from tank water and food.[42] The comparison between our study and that of Piccini et al. demonstrates that *Xenopus* microbiomes undoubtedly vary from one laboratory to another based on husbandry and other environmental variables. Until now, no

Xenopus regeneration studies have accounted for variations in microbiomes. In order to understand how commensals influence tail regeneration, it will be important in the future to determine both the source of the *Xenopus* microbiome and how it evolves at various life stages as a critical step in determining the relative contribution of microbes and genetics.

4.2 | Gram-negative LPS concentrations and/or specific genera may determine the regenerative response

As expected, raising tadpoles in a gentamicin solution resulted in altered microbiome composition, increased the proportion of Gram-positive bacteria and decreased regeneration success compared with untreated tadpoles. While we cannot entirely eliminate the possibility that gentamicin treatment introduces an antiregenerative effect separate to the reduction of pro-regenerative Gram-negative bacteria, successful rescues through the addition of LPS to the antibiotic media suggests that the antibiotics cause no significant disruption to other facets of the regeneration pathway.

Based on our results and those of our previous studies omitting antibiotics,[12,43] baseline regeneration rates among untreated refractory stage 46 tadpoles appear to be variable between sibships, ranging from 55% to 100%. It is unclear whether this is due to genetic factors, environmental factors, the presence of antiregenerative microbial taxa in the microbiome or an interplay of all three. The abundance of *Rhodobacter spp*., demonstrated here to have an antiregenerative effect, was low (just 333 reads in total across all samples). It remains to be investigated whether other taxa observed could have a similar effect. It is difficult to compare our baseline regeneration rates with those from others' studies, as parentage and antibiotic exposure of tadpoles is not always declared.

Six bacterial genera were more abundant on the skin of successful regenerators: *Pseudomonas*, *Bosea*, *Shinella*, *Chryseobacterium*, *Delftia* and *Hydrogenophaga*. Previously, we showed that a commercial preparation of *P. aeruginosa* LPS restores tail regeneration ability in antibiotic-raised stage 46 tadpoles.[12] Here, we showed that LPS isolated from *Chryseobacterium spp. XDS4* and *Delftia spp*. were also able to rescue the regeneration process in gentamicin-raised tadpoles. While we cannot rule out that innate features of the LPS from these particular taxa specially facilitates regeneration pathways, it seems unlikely, as *E. coli* LPS is also equally effective[12] (Figure S4). It is possible the total LPS load from any Gram-negative commensal (with the exception of divergent, antagonistic LPS) is sufficient to determine regenerative success in the refractory period.

In this study, *Chryseobacterium spp. XDS4*, from which LPS was obtained, was cultured from adult frogs. However, it is unclear from 16S rRNA data whether this isolate is identical to the *Chryseobacterium* detected on tadpoles. None of the six genera of note identified here, with the exception of *Pseudomonas*, was found among the top 50 genera detected on tadpoles or adults in the recent Piccini et al. study,[42] which is to date the only other such report of skin microbiota in *Xenopus* tadpoles. Although the data suggested that tadpole skin microbiomes are shaped environmentally, a lack of parental contribution was not directly determined. Here, we show that the very early tadpole microbiome is dominated by proteobacteria and that different sibships can have different genera dominating their microbiome.

The mean number of sequencing reads collected for gentamicin-treated samples was lower than for untreated samples in all sibships, but this was most pronounced in Sibship A. We sequenced DNA from 50 tadpoles per sibship/treatment, and DNA quantities were standardised by the sequencing facility both during sequencing library preparation and final pooling prior to sequencing. However, library preparation was unsuccessful for approximately one-third of gentamicin-treated Sibship A samples. The DNA in this study was extracted from whole tail samples and is thus a mixture of tadpole and microbial DNA in proportions that may vary between samples. The lower number of reads generated from the DNA of gentamicin-treated samples is consistent with a reduction of total bacterial numbers in gentamicin-treated tadpoles, with a consequent decrease in dominant Gram-negative bacteria and their LPS. Further support comes from the much higher numbers of colonies obtained from tadpole skin extracts when gentamicin was not used, although this used two different sibships. A quantitative assessment of LPS could be done in the future to test the correlation more directly.

4.3 | Commensal microbiota may have a critical role in regeneration and scar-free wound healing

While the role of individual taxa is a developing area of research, recently, evidence is emerging to support a critical role for the microbiome in regeneration and wound healing in other model organisms. In *Schmidtea mediterranea*, free-living flatworms with remarkable regeneration abilities, a pathogenic microbiome has been shown to derail regeneration.[13] *Aquitalea sp*. FJL05, a Gram-negative commensal bacterium of another planarian, *Dugesia japonica*, can dramatically affect the pattern of regeneration, resulting in worms with two heads.[14] In this case, however, indole, a small molecule produced by *Aquitalia*, rather than LPS, was the cause of the effect. Two recent studies highlight the potential role of microbiota in mouse skin and ear regeneration. Wang et al. reported that germ-free mice showed reduced levels of wound-induced hair follicle neogenesis and stem cell markers. The inflammatory cytokine IL-1β and keratinocyte-dependent IL-1R-MyD88 signalling were found to be essential for regeneration.[16] In healer MRL mice, Velasco et al. showed that healing of ear punch wounds is linked with the gut microbiome. Excitingly, this healing ability could be transferred to non-healer mice by faecal transplant.[15]

4.4 | TLR4 signalling may contribute to the regenerative response in tadpole tails

TLR4 signalling is not as well characterised in amphibia as it is in mammals. Recent work in urodele amphibia (axolotl) showed that inflammatory responses to PAMP ligands, such as LPS, through TLRs, are conserved. However, responses to Damage-Associated Molecular

Patterns (DAMPs) were found to have fundamental differences from those seen in mammals.[44] We note that orthologs of CD14 and MD2, which in mammals aid in the presentation of LPS to TLR4, appear to be absent from the *Xenopus* genomes. A third regulator of this interaction, lipopolysaccharide-binding protein (LBP), is present and would be worth targeting in the future.

Our results partially support the involvement of LPS-TLR4 in regenerative pathways suggested by Bishop and Beck.[12] Addition of LPS from *R. sphaeroides*, a known TLR4 antagonist) to antibiotic-raised tadpoles lead to reduced regeneration quality, with a similar effect seen in *X. laevis Tlr4.S* crispants. However, the inhibition of regeneration in these experiments was not absolute. *Rhodobacter sphaeroides* LPS, while achieving significant quality reduction, was not able to completely suppress regeneration, possibly due to competition for binding sites from remaining TLR4 agonist microbes. As discussed earlier, very few *Rhodobacter* sequences were detected, suggesting that *R. sphaeroides* is unlikely to be physiologically relevant in tadpole regeneration. In the CRISPR/Cas9 experiments, 100% editing was not achieved for any tadpole, despite trialling multiple sgRNAs and sgRNA concentrations to maximise efficacy. Mosaicism is an inherent problem with CRISPR/Cas9 editing and results in unedited cells within an embryo, potentially leaving a proportion of TLR4 signalling pathways intact. This would at least partially account for the persisting (albeit qualitatively poorer) regeneration capability in tadpole cohorts. Additionally, the multiple potential edits produced by any given sgRNA are unlikely to be equal in their effect on gene function (e.g., frameshifts vs. in-frame InDels). These factors taken together may go some way to explaining the lack of correlation between editing percentage and rehabilitation outcome in individual tadpoles, despite a significant correlation for the cohort taken as a whole. While direct injection of the sgRNA-Cas9 protein complex minimises mosaicism over-delivering DNA plasmids encoding sgRNA/Cas9,[45] strategies such as simultaneous use of multiple sgRNAs[46] and crossing of F_0 crispants to generate complete knockouts in F_1[47] could be used in the future to knock out *Tlr4.S* completely. Further to the above, it has been demonstrated that gene knockout can lead to up-regulation of related genes in compensation.[48] Theoretically, this would dampen the effect of TLR4 knockdown and allow some level of regeneration to proceed. A recent CRISPR/Cas9 knockdown of TGFβ1, one of the earliest players known to be required for tail regeneration[49] using three sgRNAs also demonstrated a reduced quality, delayed tail regeneration response in *X. tropicalis*[50].

TLRs have broad specificity to detect PAMPs and each receptor has its own ligand preference.[51] While TLR4 plays a central role in mediating responses to LPS, it is possible that LPS also stimulates other receptors. TLR2 may also be responsive to LPS (reviewed in de Oliviera Nascimento et al.,[52] and so it may be necessary to target TLR2 and TLR4 together to prevent LPS signalling. TLR4 can also be activated by DAMPs,[53] such as heat-shock protein HSP60 (associated with regeneration in fish[54] and frogs[55] as well as extracellular matrix components like heparan sulphate (associated with amphibian regeneration[56,57]) and tenascin C. A future approach could be to edit the gene for lipopolysaccharide-binding protein (*Lbp.L*), which may mediate TLR4 receptor-LPS ligand binding. The cytoplasmic adaptor MyD88 has been implicated in axis formation in the early development of *Xenopus*[58] and so is not a usable target.

4.5 | Conclusions

Our results demonstrate that LPS from Gram-negative bacteria enhances regenerative outcomes in *X. laevis* tadpoles and that the signalling pathway mediating this response involves TLR4, at least in part. We suggest that future studies should examine the concurrent roles of other candidate receptors using gene knockdown and also survey the individual effects of LPS from a broad range of bacterial taxa. Ultimately, this line of study has the potential to improve medicinal and veterinary outcomes in wound healing and regeneration.

AUTHOR CONTRIBUTIONS

Conceptualisation: CB and XM. Data curation: DH. Formal analysis: PC, CB and XM. Funding acquisition: CB and XM. Investigation: TD, CG, DH and CB. Methodology: CB, JW, XM and CG. Project administration: XM and CB. Supervision: XM and CB. Visualisation: CB, XM, PC and DH. Writing – original draft: PC, CB and XM. Writing – review & editing: PC, CB and XM.

ACKNOWLEDGMENTS

We are grateful for funding from the Royal Society of New Zealand Marsden Fund 19-UOO-245 to support this work. CG was partly funded by a University of Otago Master's research scholarship. Open access publishing facilitated by University of Otago, as part of the Wiley - University of Otago agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material The data that support the findings of this study are openly available in NCBI Bioproject at https://www.ncbi.nlm.nih.gov/bioproject/780297, reference number PRJNA780297.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Chapman PA, Gilbert CB, Devine TJ, et al. Manipulating the microbiome alters regenerative outcomes in *Xenopus laevis* tadpoles via lipopolysaccharide signalling. *Wound Rep Reg*. 2022;30(6):636-651. doi:10.1111/wrr.13003