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Supplementary Materials for

The lungfish cocoon is a living tissue with antimicrobial functions

Ryan Darby Heimroth, Elisa Casadei, Ottavia Benedicenti, Chris Tsuyoshi Amemiya, Pilar Muñoz, Irene Salinas*

*Corresponding author. Email: isalinas@unm.edu

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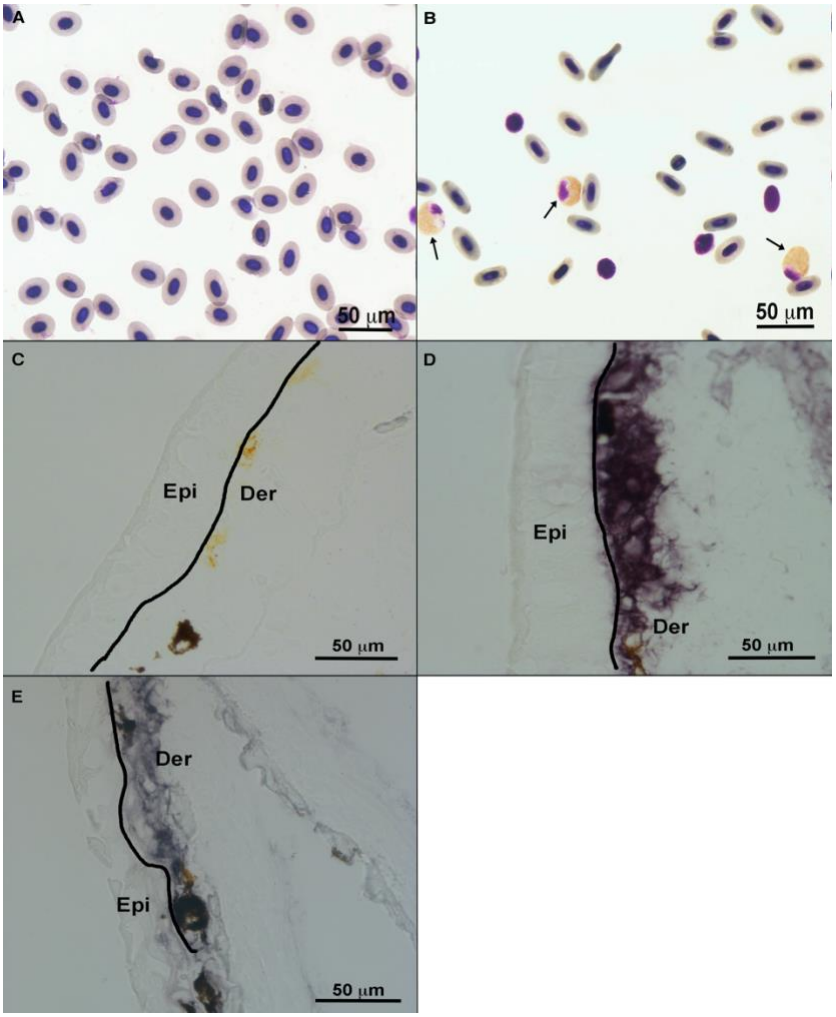

Fig. S1.

Terrestrialization results in mobilization of granulocytes into circulation and in depletion of dermal stem cells in African lungfish. **(A)** Giemsa stain of a free-swimming control lungfish blood smear. **(B)** Giemsa stain of terrestrialized lungfish blood smear. Images are representative of n = 5 per group. Arrows indicate granulocytes. **(C)** Skin cryosection of a free-swimming lungfish negative control for alkaline phosphatase staining **(D)** Skin cryosection of a free-swimming lungfish stained for alkaline phosphatase activity (purple) showing large numbers of multipotent, embryonic-like stem cells. **(E)** Terrestrialized lungfish skin cryosection stained for alkaline phosphatase activity showing reduced deposits (purple) of multipotent embryonic-like stem cells in purple. Epidermis (Epi), Dermis (Der). Images representative of n = 3 per group.

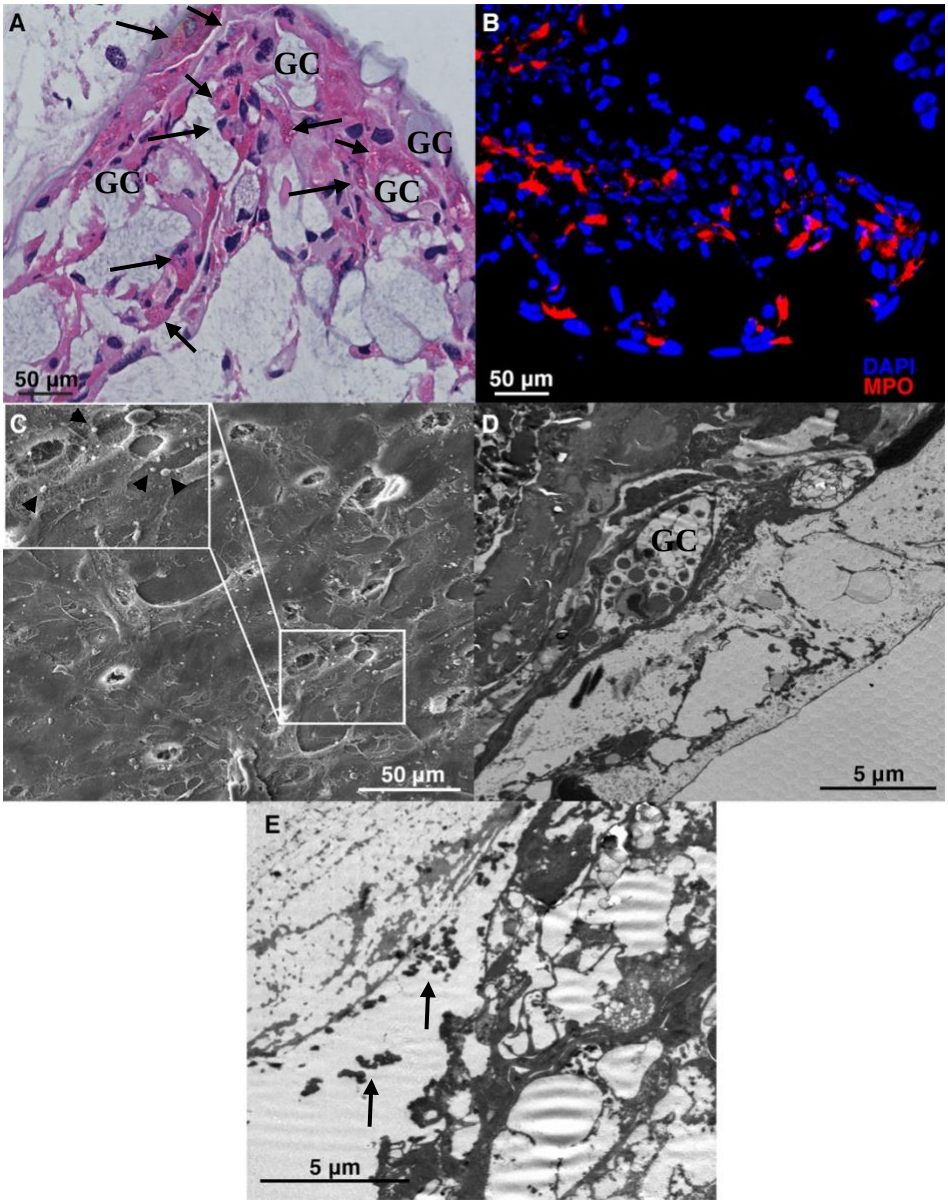

Fig. S2.

The African lungfish cocoon contains large numbers of granulocytes, goblet cells and bacteria. **(A)** Magnified H&E staining of the lungfish cocoon showing abundant granulocytes with eosinophilic granules (black arrows) and goblet cells (GCs). **(B)** Terrestrialized lungfish mucus cocoon stained with DNA stain DAPI (blue) and anti-myeloperoxidase antibody (MPO, red) confirming presence of granulocytes in the lungfish cocoon. **(C)** SEM (coronal view) of the lungfish cocoon showing bacterial cells trapped in it (black arrow heads in inset). **(D)** Transmission electron micrograph of a terrestrialized mucus cocoon sample showing presence of a goblet cell (GC) with many secretory mucus granules with dense core. **(E)** TEM of the lungfish cocoon showing large groups of bacteria (black arrows). Images are representative of n = 2-3.

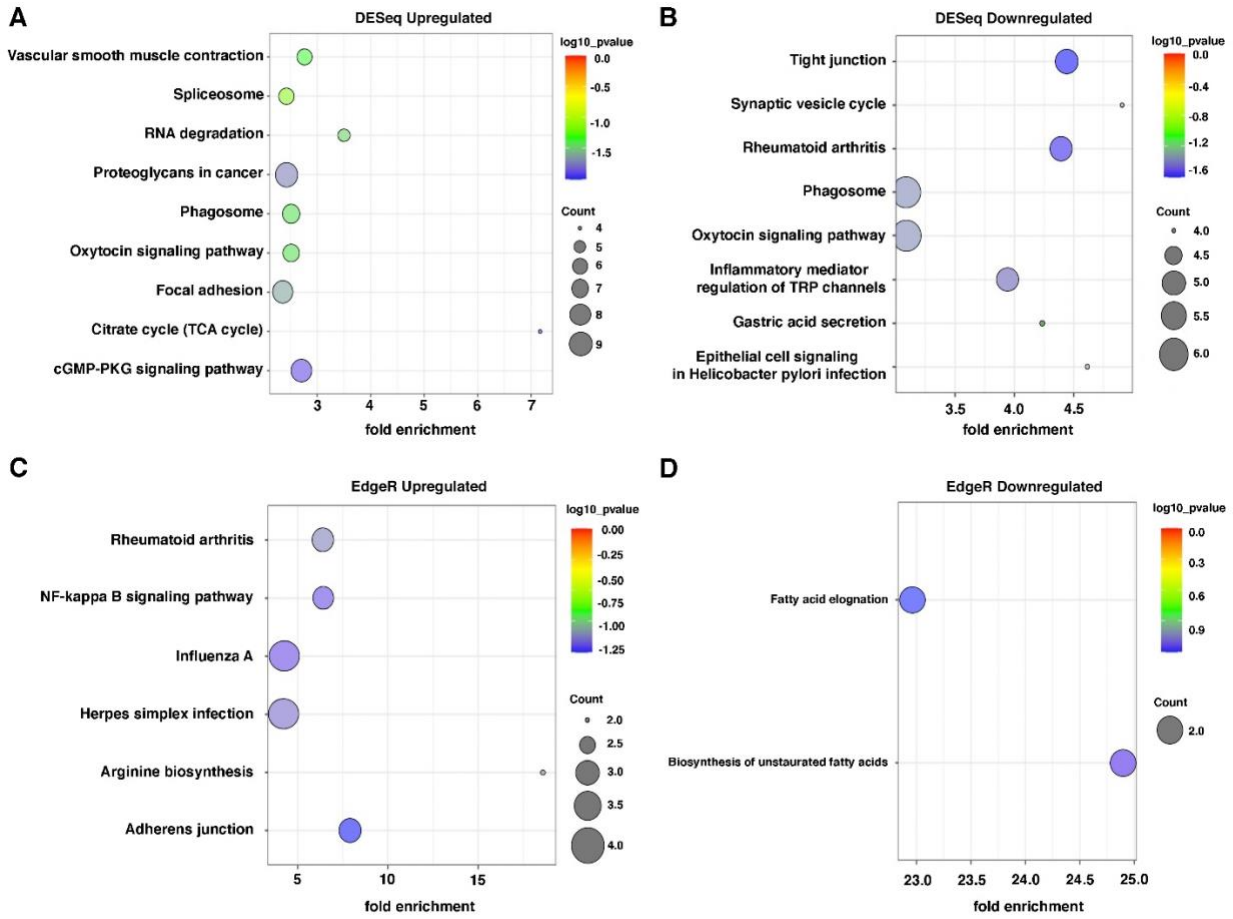

Fig. S3.

Terrestrialization results in global transcriptional changes in the African lungfish skin. Scatter plots of enriched KEGG pathways. **(A)** Up-regulated genes in the skin of terrestrialized lungfish compared to free-swimming lungfish based on DESeq analysis. **(B)** Down-regulated genes in the skin of terrestrialized lungfish compared to free-swimming lungfish based on DESeq analysis. **(C)** Up-regulated genes in the skin of terrestrialized lungfish compared to free-swimming lungfish based on EdgeR analysis. **(D)** Down-regulated genes in the skin of terrestrialized lungfish compared to free-swimming lungfish based on EdgeR analysis. The fold enrichment indicates the ratio of the expressed gene number to the total gene number in a pathway. The size and color of the points represent the gene number and the log10 p-value of each pathway, respectively. Note that no significant KEGG pathways were found in the down-regulated gene list using EdgeR **(D)**.

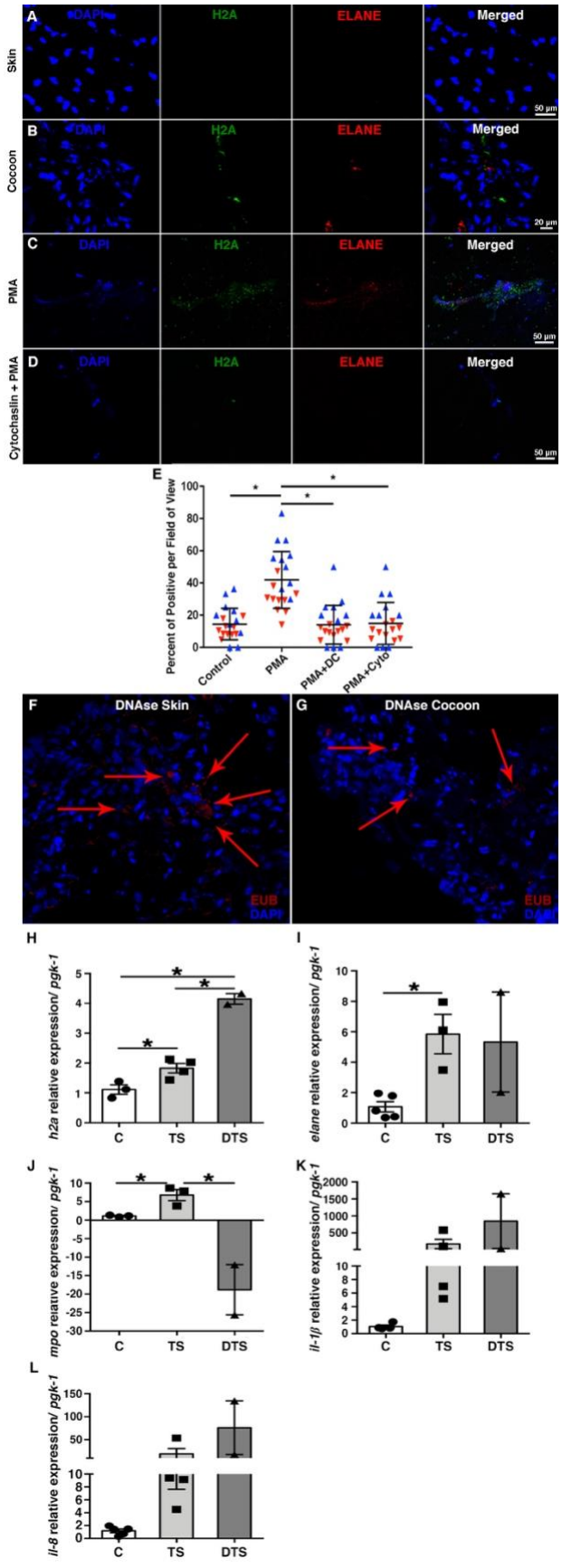
A
DAPI
H2A
ELANE
Merged
Skin
50 µm
B
DAPI
H2A
ELANE
Merged
Cocoon
20 µm
C
DAPI
H2A
ELANE
Merged
PMA
50 µm
D
DAPI
H2A
ELANE
Merged
Cytochastin + PMA
50 µm
E
Percent of Positive per Field of View
Control
PMA
PMA+DC
PMA+Cyto
F
DNAse Skin
EUB
DAPI
G
DNAse Cocoon
EUB
DAPI
H
h2a relative expression/ pgk-1
C
TS
DTS
I
elane relative expression/ pgk-1
C
TS
DTS
J
mpo relative expression/ pgk-1
C
TS
DTS
K
il-1β relative expression/ pgk-1
C
TS
DTS
L
il-8 relative expression/ pgk-1
C
TS
DTS

Fig. S4.

In vivo DNAse I treatment eliminates ETosis in the lungfish cocoon and results in increased bacterial loads and changes in immune gene expression. Representative confocal microscopy images of **(A)** terrestrialized lungfish skin cryosections and **(B)** mucus cocoon cryosections obtained from animals treated with DNase I were stained with DNA stain DAPI (blue), anti-histone 2A antibody (H2A) (green), anti-human neutrophil elastase antibody (ELANE) (red) and merged to confirm absence of ETosis. **(C)** Representative confocal microscopy images of gut cell suspensions from free-swimming lungfish stimulated with PMA used as positive ETosis controls. Cells were stained with DNA staining (DAPI), anti-histone 2A antibody (H2A) (green), anti-human neutrophil elastase antibody (ELANE) (red) and merged. **(D)** Representative confocal microscopy images of gut cell suspensions from free-swimming lungfish stimulated with PMA+Cyto. Cells were stained with DNA staining (DAPI), anti-histone 2A antibody (H2A) (green), anti-human neutrophil elastase antibody (ELANE) (red) and merged. **(E)** Quantification of the percentage of cells in gut cell suspensions (control, stimulated with PMA, stimulated with PMA+DC or stimulated with PMA+Cyto) from free-swimming lungfish. Each symbol corresponds to one field of view. Each color indicates a different animal. **(F)** Maximum projection (1 μm-thick Z-stacks, 5 stacks) of confocal fluorescence images of a DNAse I treated lungfish skin cryosection labeled with EUB338 oligoprobe (red). **(G)** Maximum projection (1 μm-thick Z-stacks, 5 stacks) of confocal fluorescence images of a DNAse I treated lungfish cocoon cryosection labeled with EUB338 oligoprobe (red). Note the high numbers of bacteria in the skin of DNAse I treated animals (n = 3). Cell nuclei were stained with DAPI (blue). Red arrows indicate bacterial cells. **(H-L)** Quantification of gene expression levels of *h2a*, *elane*, *mpo*, *il1b* and *il8* in control free-swimming lungfish skin (C), terrestrialized lungfish skin (TS), and DNase I treated terrestrialized lungfish skin (DTS) (n = 3-5/group). Gene expression levels were normalized to the house-keeping gene *pgk-1*. Data were analyzed by unpaired Student's *t*-test. $*P < 0.05$.

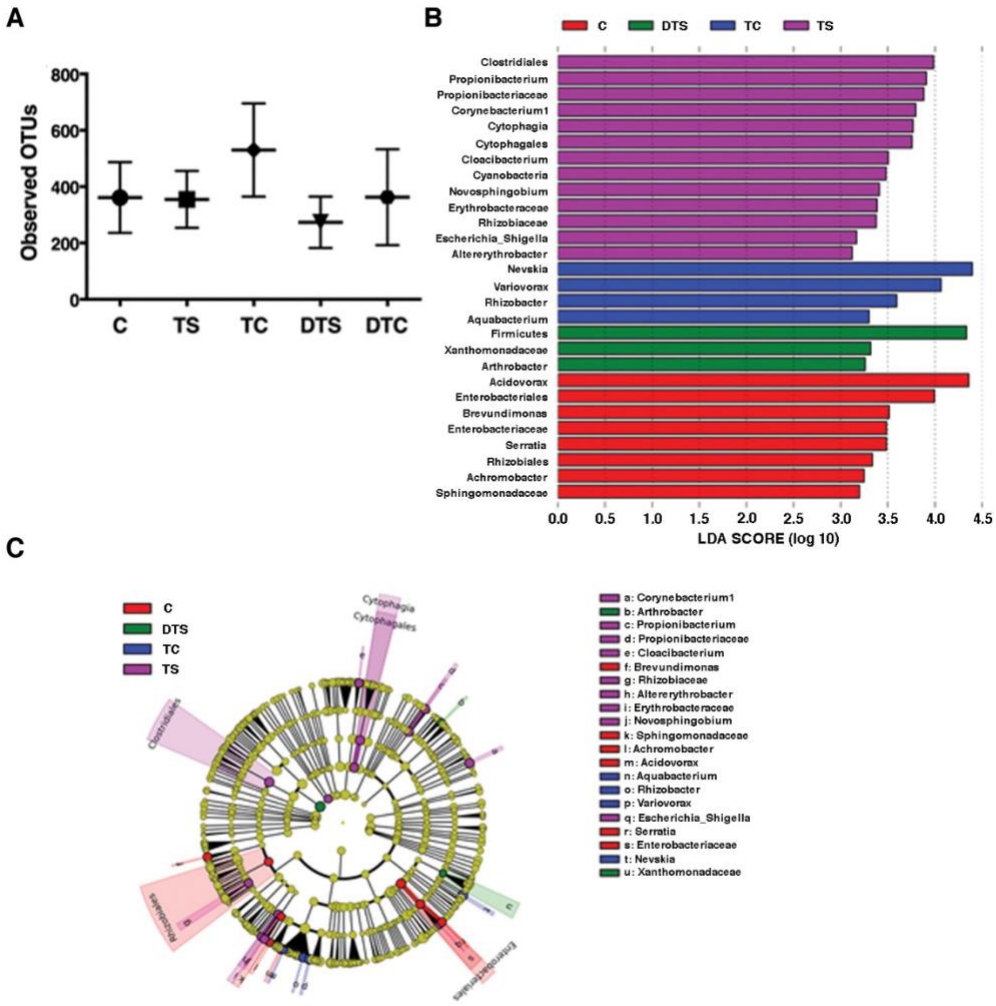

Fig. S5.

Microbiome analyses of the lungfish skin reveals unique bacterial communities in free swimming and terrestrialized animals and the effects of extracellular DNA removal. Microbiome analyses of lungfish control skin (CS), terrestrialized skin (TS), cocoon (TC), DNase I treated skin (DTS) and DNase I treated cocoon (DTC) (n=3-4). **(A)** Mean number of observed OTUs in each sample. **(B)** Bar chart of the log-transformed linear discrimination analysis (LDA) score of bacterial taxa found to be significantly associated with CS, TS, TC and DTS by LEfSe ($P < 0.05$). No taxa were uniquely associated with DTC. **(C)** Cladogram representation of LEfSe analysis showing bacterial taxa that were significantly associated with each of the tissue samples ($P < 0.05$).

Genes	**Sequences (5'-3')**
*β-actin*_F0	GCCTCTGGTCGAACAACTGG
*β-actin*_R0	GGAGGATGCAGCAGTAGCCA
*h2a*_F1	CGGACATGATTATTGCAGCTAT
h2a _R1	GCTTTTGGCTTGGATGTCTTT
*csta*_F1	GGTACTTCTGAAGTCAAGCCTG
*csta*_R1	GGATGTTATGCAAACTTACTTCTT
*cxcr2*_F1	GCAAAGACAGTAATATTGTTTGG
*cxcr2*_R1	GCATCAGTAACAGACAAATGG
*il1b*_F0	CCACCTGTCTATGTACAAATCACG
il1b _R0	GCAGACTCAAACCTGGAGGAG
*il8*_F0	CCTCCTGTGCTGTGTGACTGT
*il8*_R0	GGAACTTTCCAACATTCTGTCG
*mpo*_F1	GCCCAACTGCAGATCATAACG
*mpo*_R1	GGTGCTCTTGGAAGGCTTCA
*defb1*_F1	GGAGTTATTGGTGAAAAAGATGG
*defb1*_R1	GCAGATCTTCTACGTACACAACACC
*defb2*_F1	GGAATAATCAGTGAACAAGATGGAG
*defb2*_R1	GGAATCATTTAGGTGTGCAGCA
*defb3*_F1	GGTGCATTTCAAAAGTATCGAGACAC
*defb3*_R1	GTTTTGGTGTGCAGCATCTCC
*defb4*_F1	GGAGTTAATGTGGAGGCTCATT
*defb4*_R1	CCAAGTTCTTCATGTTAGACAGC
muc2_F0	GCTGTTTCCATTACGAATGTGAAG
*muc2*_R0	CCTCTGTGTGGTACAAATCCTTC
*muc4*_F0	GGAAATGGGACTCCTTTTATGAC
*muc4*_R0	CCTGAGCTAGTGGAGATTCCTTG
ck8_F1	GCTGAACTAACCAGGTACATC
ck8_R1	GGTGGCAATTTCAATGTCTAGG
pgk-1_F1	GCAAAACAGATTGTATGGAATGGA
pgk-1_R1	GGCACAGCATGTGGCTGTATC
341F	CCTACGGGNGGCWGCAG
805R	GACTACHVGGGTATCTAATCC

Table S1.

Primers used in this study for RT-qPCR.