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New Tick Defensin Isoform and Antimicrobial Gene Expression in Response to *Rickettsia montanensis* Challenge▽

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Recent studies aimed at elucidating the rickettsia-tick interaction have discovered that the spotted fever group rickettsia *Rickettsia montanensis*, a relative of *R. rickettsii*, the etiologic agent of Rocky Mountain spotted fever, induces differential gene expression patterns in the ovaries of the hard tick *Dermacentor variabilis*. Here we describe a new defensin isoform, defensin-2, and the expression patterns of genes for three antimicrobials, defensin-1 (*vsnA1*), defensin-2, and lysozyme, in the midguts and fat bodies of *D. variabilis* ticks that were challenged with *R. montanensis*. Bioinformatic and phylogenetic analyses of the primary structure of defensin-2 support its role as an antimicrobial. The tissue distributions of the three antimicrobials, especially the two *D. variabilis* defensin isoforms, are markedly different, illustrating the immunocompetence of the many tissues that *R. montanensis* presumably invades once acquired by the tick. Antimicrobial gene expression patterns in *R. montanensis*-challenged ticks suggest that antimicrobial genes play a role during the acquisition-invasion stages in the tick.

In natural transmission cycles, the vector-pathogen interaction is of central importance with respect to sylvatic epizootic and enzootic cycles (vector-pathogen-nonhuman animal), as well as zoonotic cycles that involve humans as incidental hosts (vector-pathogen-human). Vector-pathogen interactions are studied in many contexts, including how vectors respond to microbial challenge. Investigating vector innate immunity addresses the broad question of what factors intrinsic to the arthropod underlie vector competence.

The innate immune system of ticks is less well studied than those of insects. Most reports deal with antimicrobial blood meal digestion by-products (9, 32, 44) and differential patterns of expression of antimicrobials such as lectins (22), lysozymes (21, 42), and defensins (4, 18, 23, 33–35, 39).

Defensin expression is reported to occur in the midguts, fat bodies, hemolymph, and hemocytes of both argasid (soft) and ixodid (hard) ticks as well as the synganglia of ixodid ticks. Before this study, only one defensin isoform, functional against gram-positive bacteria, was isolated from the plasma of the hard tick *Dermacentor variabilis* (18). Further research implicated hemocytes as one source of the soluble peptide (4). Two nonionic defensin isoforms, ADP1 and ADP2, that originate from synganglia of the hard tick *Amblyomma hebraeum* have been identified and found to possess activity against gram-positive and gram-negative bacteria but not against the fungal pathogens *Candida albicans* and *Candida glabrata* (23). Additionally, there are numerous studies reporting defensin-like genes in *Ixodes* sp. (13, 39, 47).

From previous work, we know that *D. variabilis* is capable of expressing antimicrobial genes in response to *Borrelia burgdorferi* (16, 19), that the abundance of transcripts of immune responsive factor D (43) and lysozyme (42) genes increases upon challenge with *Escherichia coli*, and that a pattern of differential gene expression (including the expression of immune response genes) occurs in *Rickettsia montanensis*-infected ovaries (27, 28). Therefore, we hypothesized that differential levels of antimicrobial gene expression in the midgut and fat body would occur as a result of *R. montanensis* challenge. To test our hypothesis, we challenged *D. variabilis* ticks with *R. montanensis*, an obligate intracellular bacterium that infects ticks in nature, by using the capillary feeding technique and assessed antimicrobial gene expression in the midguts, an important first barrier to bacterial infection, and the fat bodies, tissue involved in the systemic response of the immune system.

In this study, we report a second, phylogenetically distinct defensin-like isoform from *D. variabilis* and provide antimicrobial gene expression profiles corresponding to defensin-1 (*vsnA1*), defensin-2 (this study), and lysozyme in response to per os challenge with *R. montanensis*. We also present a statistical pipeline that relies on hypothesis testing for the analysis of small quantitative reverse transcriptase PCR (qRT-PCR) data sets.

MATERIALS AND METHODS

Ticks and rickettsia. An *R. montanensis*-free *D. variabilis* tick line was maintained at Old Dominion University as previously described (17). All animals were handled according to Old Dominion University's Institutional Animal Care and Use Committee regulations. For gene expression experiments, female ticks were allowed to feed for 4 days on New Zealand White rabbits (*Oryctolagus cuniculus*) and were then forcibly removed for analysis. Naïve rabbits were used for feeding to maintain an *R. montanensis*-free line. *R. montanensis* was grown on a subconfluent L929 mouse fibroblast cell line in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum at 34°C and 5% CO_2. At 5 days postinoculation, the cells were harvested by trypsinization, resuspended in 5 ml

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TABLE 1. qRT-PCR primers used in this study

Gene product	Primer[a]	Primer sequence	Amplicon size (bp)
Actin	AZ3515 (F)	5′-CCGGTTCAGCCCTCGTTCT-3′	266
	AZ3514 (R)	5′-TTGAGGCCAGGGATGGAGC-3′	
Defensin-1	AZ3519 (F)	5′-CTTTGCATCTGCCTTGTCTTTCTC-3′	214
	AZ3518 (R)	5′-AATTCCTGTAGCAGGTGCAGG-3′	
Defensin-2	AZ3517 (F)	5′-ATGAGGTTCACCTCTGCCATCTTC-3′	234
	AZ3516 (R)	5′-TTGTCATCAGACTGGTCCAGCG-3′	
Lysozyme	AZ3911 (F)	5′-GATTGGATCTGCTTGGCAACAGC-3′	297
	AZ3912 (R)	5′-TCAATATCGGCACCCCTTGACG-3′	
R. montanensis-specific 17-kDa protein	AZ3257 (F)	5′-TACAGGCACACTTCTTGGCG-3′	339
	AZ3258 (R)	5′-GCTTTTTGTTGTTTTCCGC-3′	
R. montanensis-specific *gltA* protein	AZ4002 (F)	5′-ATTGCCTCACTTTGGGGACCTG-3′	427
	AZ4003 (R)	5′-TCCATTGTGCCATCCAGCCTAC-3′	

[a] F, forward; R, reverse.

of fresh medium, collected by centrifugation at 500 × *g* for 5 min, and resuspended in 5 ml of fresh whole sheep's blood.

Sequence retrieval and bioinformatic analysis of defensin-2. Defensin-2 was originally discovered by using an expressed sequence tag approach in our laboratory (42). Our characterization of defensin-2 is a continuation of that work. Defensin-1 and lysozyme from *D. variabilis* have both been reported previously (18, 42); however, defensin-2 has not yet been formally reported. The deduced amino acid sequence for defensin-2 was retrieved using the ExPASy sequence retrieval system (http://au.expasy.org/srs5). The sequences of defensin-2 and other tick defensins were retrieved using the following accession numbers: *D. variabilis* defensin-2, Q6YC89; *D. variabilis* defensin-1, Q86QI5; *Amblyomma hebraeum* defensin-1, Q5VJF9; *Amblyomma hebraeum* defensin-2, Q5VJF8; *Argas monolakensis* defensin, Q09JE6 and Q09JJ7; *Ornithodoros moubata* defensin A, Q9BLJ3; *Ornithodoros moubata* defensin B, Q9BLJ4; *Orithodoros moubata* defensin C, Q8MY08; *Ornithodoros moubata* defensin D, Q8MY07; *Ixodes ricinus* preprodefensin-2 (fragment), Q2HYY9; *Ixodes ricinus* preprodefensin (fragment), Q7YXK5; *Ixodes scapularis* salivary gland putative secreted protein, Q5Q979; *Ixodes scapularis* scapularisin preproprotein, Q5ISE3; *Haemaphysalis longicornis* antimicrobial peptide, Q58A47; and *Boophilus microplus* preprodefensin, Q86LE4. Sequences were retrieved in FASTA format and aligned using MUSCLE (6, 7), available through the European Bioinformatics Institute website toolbox (http://www.ebi.ac.uk/Tools/sequence.html). Alignments were edited using GeneDoc version 2.6.003 (http://www.psc.edu/biomed/genedoc/). Global pairwise alignments of defensin-1 and tick defensins were performed using Align accessed through the European Bioinformatics Institute toolbox (http://www.ebi.ac.uk/Tools/sequence.html). Each protein sequence was submitted to InterProScan (http://www.ebi.ac.uk/InterProScan/) to search for the presence of a putative domain structure(s). SignalP (http://www.cbs.dtu.dk/services/SignalP/) analysis of defensin-2 was also performed to search for signal sequences. Additionally, an analysis of the peptide domains of each tick defensin was performed using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/).

Tick challenge and dissection. To infect ticks with *R. montanensis*, ticks were fixed to double-sided sticky tape and 10-μl microcapillaries were placed over the mouthparts. Microcapillaries were filled with either *R. montanensis*-infected L929 cells (rickettsia-challenged group) or uninfected L929 cells (control group) resuspended in whole sheep's blood. Ticks were incubated at 22 to 25°C with 80% humidity while feeding. The capillaries were replaced daily with new capillaries and the appropriate blood meal. At each time point, ticks were removed from the tape and washed in 70% ethanol and 1× phosphate-buffered saline (PBS). Tissue dissections were performed while each tick was submerged in 1× PBS. Briefly, a transverse cut was made to sever the head and posterior alloscutum, a continuous cut was made along the perimeter of the alloscutum, and the resulting flap was removed to gain access to the organs. Our dissections of the fat bodies were not 100% efficient because fat-body cells are interspersed throughout the tracheolar network and it is difficult to obtain pure fat-body tissue. Therefore, we note that our fat-body tissue included tracheolar tissue. Each set of organs from each tick was washed in three changes of 1× PBS, and the sets were stored separately at −80°C in RLT lysis buffer (supplemented with 0.143 M β-mercaptoethanol) from the RNAeasy Micro Total RNA isolation kit (QIAGEN). Total RNA was purified using the RNAeasy Micro Total RNA isolation kit as described by the manufacturer (QIAGEN) for animal tissues. Only those ticks that could be visibly verified as having imbibed a blood meal (by examination of the gut and evidence of continued defecation over the feeding period) were processed for further analysis.

Relative quantitative RT-PCR conditions. For defensin, lysozyme, and actin genes, standard target DNA was amplified from recombinant plasmid, gel purified, and quantified using a spectrophotometer. The primer design and melting temperature were optimized for each gene amplification. (i) Primers were designed to amplify a 400-bp region of each gene with the aid of MacVector version 7.1. Primer pairs were chosen based on a theoretical melting temperature of 55°C and the guidelines set forth in Stratagene's *Introduction to Quantitative PCR: Methods and Application Guide* (45). (ii) A melting temperature gradient procedure using the standard PCR cycling conditions described above was performed, and the melting temperature that provided a single product without visible primer dimers was chosen for use in qRT-PCR experiments. Table 1 lists all of the primers used in this study. Two hundred nanograms of template RNA (purified as described above) was reversed transcribed and amplified using one-step brilliant SYBR green master mix (Stratagene, La Jolla, CA) on an MX3000 Stratagene real-time thermal cycler. Cycling conditions included one cycle of 50°C for 1 h; one cycle of 95°C for 10 min; and 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s followed by a disassociation cycle of 95°C for 1 min, a 30-s hold at 55°C, and a ramp up at 0.1°C/s to 95°C for a 30-s hold. No reverse transcriptase and no template control reactions were run to account for genomic DNA and reagent template contamination, respectively, for each template and primer pair. Cycle threshold (C_T) data were imported into an Excel spreadsheet, which facilitated organization and statistical analysis. The amplification efficiency for each antimicrobial gene was determined using standard curve data that were imported into LinRegPCR (37). Briefly, each standard DNA template was diluted in a series from 10^{10} down to 10^3 copies μl^{-1}. Fluorescence data representing the sigmoidal curve for each amplification in the dilution series were collected. The fluorescence data were imported into LinRegPCR for calculation of the amplification efficiency. The average efficiency for the dilution series for each gene was used to derive normalized gene expression values. Gene expression was calculated as the efficiency-corrected median normalized expression by using the following formula: (29)

$$\mathrm{NE} = \frac{E_{(\mathrm{target})}^{C_{T(\mathrm{target})}}}{E_{(\mathrm{reference})}^{C_{T(\mathrm{reference})}}} \quad (1)$$

where NE is the normalized expression value, E_{target} and $E_{\mathrm{reference}}$ represent the efficiencies for the target and the reference, the target is defensin-1 or -2 or lysozyme, and the reference is actin. Individual ticks were considered replicates in this study, i.e., one tick equaled a replicate. Normalized expression values were calculated separately for each replicate (each target with its respective reference), and the median normalized expression was calculated as the central point estimator for each tissue studied, time point assayed, and feeding status. Outliers were identified using the interquartile range and removed. Experimental median values for each group are represented by n_1 (challenged or fed) and n_2 (control or unfed). For easy visualization of the trends, experimental medians were plotted as the median normalized expression versus the time post-experimental feeding (see Fig. 4). Gene expression studies report differences between groups as ratios, which express the direction (up [+] or down [−]) and the magnitude of change. Therefore, statistical analyses were designed based on the difference

(*n*-fold; FD) between results for the challenged and control or fed and unfed ticks. The experimental FD was calculated as n_1/n_2.

qRT-PCR statistical analysis: difference (*n*-fold). A critical analysis of data may reveal underlying trends not detectable by robust nonparametric statistics such as the Mann-Whitney *t* test or Kruskal-Wallis analysis of variance. Using the theory behind hypothesis testing, we developed a statistical pipeline where the difference (*n*-fold) between groups was reported with statistical confidence, thereby removing the convention of reporting a group difference based on an arbitrary threshold of ≥2. We formulated a 95% population confidence interval that accounts for biological and technical variability within our system. An in-depth description of how the populations were derived and of the statistical calculation and notation is provided in the appendix. The hypothesis tests were based on the following null (H_o) and alternative (H_a) hypotheses:

$$H_o\text{: FD} = \text{FD}''$$

$$H_a\text{: FD} \neq \text{FD}''$$

FD is the experimental difference (*n*-fold) described above, and FD″ is the population difference (*n*-fold) calculated as $\hat{n}_1/\hat{n}_2$, where $\hat{n}_1$ is the median for one population (challenged or fed ticks) and $\hat{n}_2$ is the median for the other population (control or unfed ticks). FD was said to support a group difference at the experimental level, n_1/n_2, if the experimental 95% confidence interval failed to include 1 (indicating no change) (10). This criterion is represented by the following equations:

$$\left(\frac{n_1}{n_2}\right)\exp[\pm 1.96(\text{var}N_1 + \text{var}N_2)^{1/2}] > 1$$

or

$$\left(\frac{n_1}{n_2}\right)\exp[\pm 1.96(\text{var}N_1 + \text{var}N_2)^{1/2}] < 1$$

where N_1 represents the population values for the challenged or fed group, N_2 represents the population values for the control or unfed group, varN_1 is the variance in N_1, and varN_2 is the variance in N_2. Experimental FDs are said to indicate statistically significant trends for rejection of H_o if one of the following is true:

$$\text{FD} > \left(\frac{\hat{n}_1}{\hat{n}_2}\right)\exp[\pm 1.96(\text{var}N_1 + \text{var}N_2)^{1/2}]$$

or

$$\text{FD} < \left(\frac{\hat{n}_1}{\hat{n}_2}\right)\exp[\pm 1.96(\text{var}N_1 + \text{var}N_2)^{1/2}]$$

Phylogenetic analysis. (i) Taxon sampling and alignment. We downloaded 111 described or putative defensin-like protein sequences from GenBank in FASTA file format. We added our newly generated putative defensin sequence from *D. variabilis* to this file and aligned the sequences locally using default parameters in the command-line version of the program MUSCLE (6, 7). This initial alignment was evaluated manually with reference to published structures of arthropod defensins (18, 34, 35), and it was determined that the conserved regions corresponding to the 3′ halves of the sequences, which contain six conserved cysteine residues, were not well homologized across the data set. We then divided the sequences into two sections, the variable preprotein region corresponding to the 5′ region and the conserved six-cysteine-containing region corresponding to the 3′ region, and aligned these two sections separately in MUSCLE by using default parameters. Resulting alignments were again visualized and manually adjusted based on conserved features of arthropod defensins. These two files were then concatenated and converted to Nexus format by using the program seqConverter.pl version 1.1, by O. Bininda-Emonds (2006).

(ii) Phylogeny estimation. The final Nexus file was analyzed under parsimony in the program PAUP* version 4.10 (Altivec) (46). We performed 500 heuristic searches, saving only the 50 best trees per replicate. Branch support was measured by using the bootstrap method (8), sampling 50 replicates with 10 trees saved per replicate, and retaining groups recovered with 50% or greater frequency. We also analyzed the data under maximum likelihood by using Bayesian inference in the program MrBayes version 3.1.2 (12, 38). We used the command "prset aamodelpr = mixed" to allow the mcmc chain to integrate over the 10 fixed amino acid substitution matrices in MrBayes. Three analyses starting from randomly different sampling spaces (seeds) were implemented. Three million iterations were performed, with a sampling from the posterior distribution completed every 100th generation. To ensure that sampling was adequately exploring the parameter space, the degree of convergence in tree topologies, clade posterior probabilities, and parameter posterior probabilities across all analyses were analyzed with the program Tracer version 1.2 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer), which provides graphical plots and numeric reports of the estimated sample size. Once burn-in plots were determined, trees along the tails of the asymptotic curves were combined from all three analyses and a consensus tree was constructed, with branch support determined by the frequency of recovered clades in the posterior distribution of trees (posterior probability).

Nucleotide sequence accession number. The sequence determined in this study has been deposited in GenBank with accession number AY159879.

RESULTS

Defensin-2 is unique with respect to primary structure and phylogenetic placement among reported arthropod defensins. While defensins are ubiquitous, recent reports have implicated defensins as multifunctional peptides (20). Indeed, the diversity in primary structure is directly correlated to the diversity in function. Therefore, we were interested in a comparison of the primary structure of defensin-2 with those of other tick defensins as well as its relationship, at the peptide level, with other arthropod defensins. By comparing the sequence of defensin-2 to those of the known mature peptides of other tick defensins (e.g., defensin-1) in a multiple alignment, we were able to predict the putative mature peptide of defensin-2 (Fig. 1). When pairwise alignments were performed to compare defensin-2 to other tick defensins, the percent similarity ranged from 23.3 to 47.4% over the entire region corresponding to the open reading frame (Fig. 1). However, a level of similarity between 35.7 and 48.7% over the deduced mature peptide was observed. Each of the tick defensins displayed in the alignment were identified as belonging to the scorpion toxin-like superfamily of proteins that comprises peptides and proteins that possess a knottin fold. The knottin fold is characterized by a beta-hairpin with two adjacent disulfide bonds. The scorpion toxin-like superfamily is composed of six families, one of which is the arthropod defensin family. InterProScan results for each of the tick defensins placed them in the arthropod family of defensins. Each possesses the six conserved cysteines that are responsible for maintenance of the αβ fold tertiary structure in solution (Fig. 1). Interestingly, architecture analysis using the Simple Modular Architecture Research Tool shows that none of the tick defensins appear to possess the knottin domain. Furthermore, the knottin domain seems to be restricted to the insect defensins. From the phylogenetic analysis, there is little distinction between the defensins of Acari, Scorpiones, and several species of Insecta in the families Lepidoptera, Diptera, and Coleoptera (Fig. 2). The majority of sampled insect defensins, however, do appear to be derived in relation to this cluster of mostly noninsect ancestral defensins. The defensins from *Amblyomma* sp. are ancestral to all other Acari defensins and group closest to those of the primitive Xiphosura defensins. Interestingly, aside from *Amblyomma* sp. defensins and defensin-2, all sampled acarine defensins contain the conserved RVRR motif that separates the variable peptide corresponding to the 5′ region from the conserved six-cysteine-containing region corresponding to the 3′ end (Fig. 2). From this observation, it is tempting to conclude that defensin-2 is ancestral to defensin-1 of *D. variabilis*; however, the lack of phylogenetic resolution prevents us from making this claim. A consistent lack of monophyly among any of the major arthropod taxa likely relates to the limited number of sampled char-

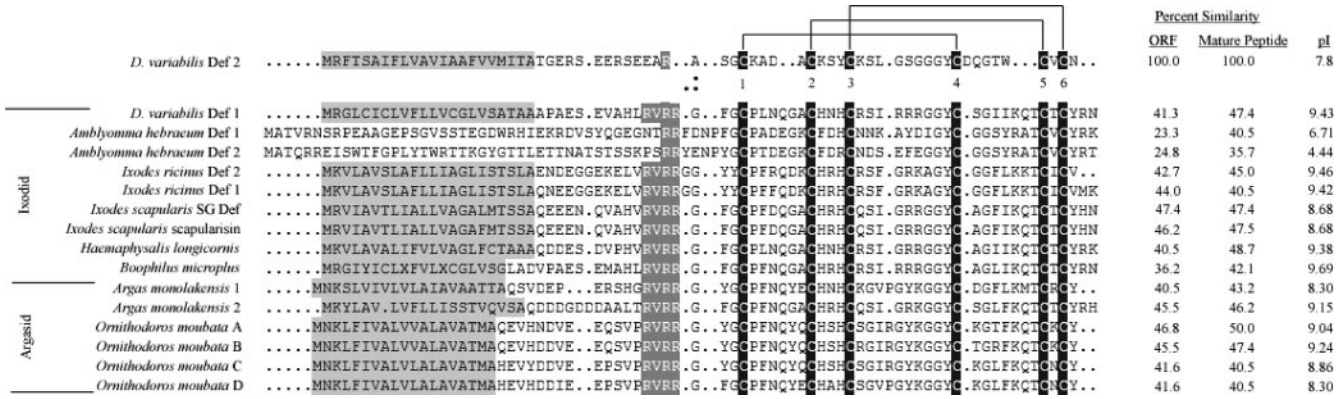

FIG. 1. Amino acid alignment among tick defensins (Def). Alignment was performed using MUSCLE. The sequences are segregated into ixodid (hard) and argasid (soft) tick defensins. Light-gray highlighting indicates the signal peptide as determined using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Dark-gray highlighting indicates a conserved region, RVRR, that separates the leader sequence from the mature peptide. The conserved cysteine residues are highlighted in black, and the cysteine bonding pattern that is characteristic of arthropod defensins is shown. The percentages of similarity for the defensin gene open reading frame (ORF) and the mature peptide are derived from pairwise alignments performed using Align and defensin-2 as the reference. The isoelectric points as predicted using the ExPASy pI/molecular weight tool are also provided. ●, amino acid 1 of mature *Amblyomma* sp. peptide; :, amino acid 1 of mature peptide of remaining tick defensins.

acters in the phylogeny estimation, although it cannot be ruled out that the sampled defensins comprise paralogous copies of peptides from similar families that have arisen from duplication events.

Multiple tissues are capable of antimicrobial gene expression. Rickettsiae presumably infect many tissues within the open circulatory system of the tick. Furthermore, the tissues responsible for antimicrobial gene expression may secrete the peptide or protein into the hemolymph, the connective tissue of the open circulatory system, making it difficult to discern the responsible tissue. For these reasons, it was of interest to determine tissue distribution patterns for antimicrobial gene expression in fed, uninfected ticks. Defensin-1 and defensin-2 had distinct tissue distribution patterns. Defensin-1 was expressed almost exclusively in the hemocytes, whereas defensin-2 was expressed mostly in the ovary, midgut, and fat body (Fig. 3A and B). Interestingly, there was little expression of defensin-2 in the hemocytes (Fig. 3B). The highest level of lysozyme expression occurred in hemocytes and midgut tissue (Fig. 3C). From the data, it can be stated that as rickettsiae migrate throughout the tick, they encounter tissues with the ability to express antimicrobial peptides.

Feeding results in differential antimicrobial gene expression patterns. Blood feeding has been observed to cause an increase of defensin and lysozyme expression in many ticks and insects (11, 31, 34, 39). To assess the effects that feeding had on antimicrobial expression in this study, we compared the levels of expression of each antimicrobial in the midguts and fat bodies from unfed and partially fed female ticks. A significant difference between the results for midguts from fed and unfed groups was observed for defensin-1 (+35.34-fold; $P = 5.812 \times 10^{-12}$) and defensin-2 (+5.140-fold; $P = 2.091 \times 10^{-6}$) (Table 2). No significant difference in the level of expression of either defensin-1 (+1.192-fold; $P = 0.0893$) or defensin-2 (+1.005-fold; $P = 0.9787$) in the fat bodies was observed (Table 2). Alternatively, lysozyme expression was observed to be significantly lower in the fat bodies from fed ticks (−17.091-fold; $P = 0.0138$) and unchanged in the midguts (−1.654-fold; $P = 0.0912$) (Table 2). As in other arthropods, in ticks, feeding alone results in differential antimicrobial gene expression patterns in the midgut and fat body.

Rickettsial challenge results in differential antimicrobial gene expression patterns. Recent reports indicate that genes putatively functioning as receptor/adhesion and immune response effectors are differentially expressed in ovaries from *D. variabilis* ticks chronically infected with *R. montanensis* (26, 28). Therefore, it was of interest to examine the transcriptional profiles of three important antimicrobial genes in response to rickettsial challenge. We used qRT-PCR to determine the percentage of ticks with a detectable *R. montanensis* challenge. The following results were obtained at the indicated time points postfeeding: 18 h, 0% of ticks; 24 h, 50% of ticks; 48 h, 50% of ticks; and 72 h, 100% of ticks were detectably challenged. *R. montanensis* was not detected in control ticks that were fed whole sheep's blood spiked with uninfected L929 cells.

(i) Defensin-1. There was an overall increase in defensin-1 gene transcript levels in the midguts of both the challenged and control groups over time (Fig. 4A; Table 3). Transcript abundance in the challenged group was 2.5-fold lower ($P = 0.0022$) than that in the control group at 18 h post-experimental feeding but became significantly higher than that in the control at 24 and 48 h post-experimental feeding (Fig. 4A; Table 3). Levels of transcript abundance in the fat bodies differed significantly between the challenged and control groups at all time points assayed (Table 3). Interestingly, defensin expression in the challenged group lagged behind that in the control group at 18 and 24 h post-experimental feeding but increased significantly over that in the control group by 48 h post-experimental feeding (Fig. 4B; Table 3).

(ii) Defensin-2. Defensin-2 gene expression in the midguts from challenged groups was significantly different from that in control groups at 18, 24, and 72 h post-experimental feeding (Fig. 4C; Table 3). As observed for defensin-1 gene expression in the fat bodies from rickettsia-challenged ticks, a significant negative difference in defensin-2 transcript abundance between groups at 24 (−2.472-fold; $P = 0.0060$) and 48 (−2.090-

FIG. 2. Estimated phylogeny of 112 defensin or defensin-like proteins from arthropods. The tree topology is that of a strict consensus tree constructed from the results of a parsimony analysis. Branch support values are from bootstrapping (left) and posterior probability (right) from a separate Bayesian analysis performed on the same alignment (see the text). The dashed box identifies the more ancestral arthropod defensins. Short sequences containing the RVRR motif between the regions of the defensins corresponding to the 5′ variable region and the 3′ conserved region are shown at each terminal, with a structural logo (5, 40) depicting the consensus of this sequence across the alignment (inset). Taxa missing this conserved sequence are indicated with a dark circle (●). Brown highlighting indicates defensin-2. Dark-gray highlighting indicates the presence of the conserved RVRR motif or the variations across taxa.

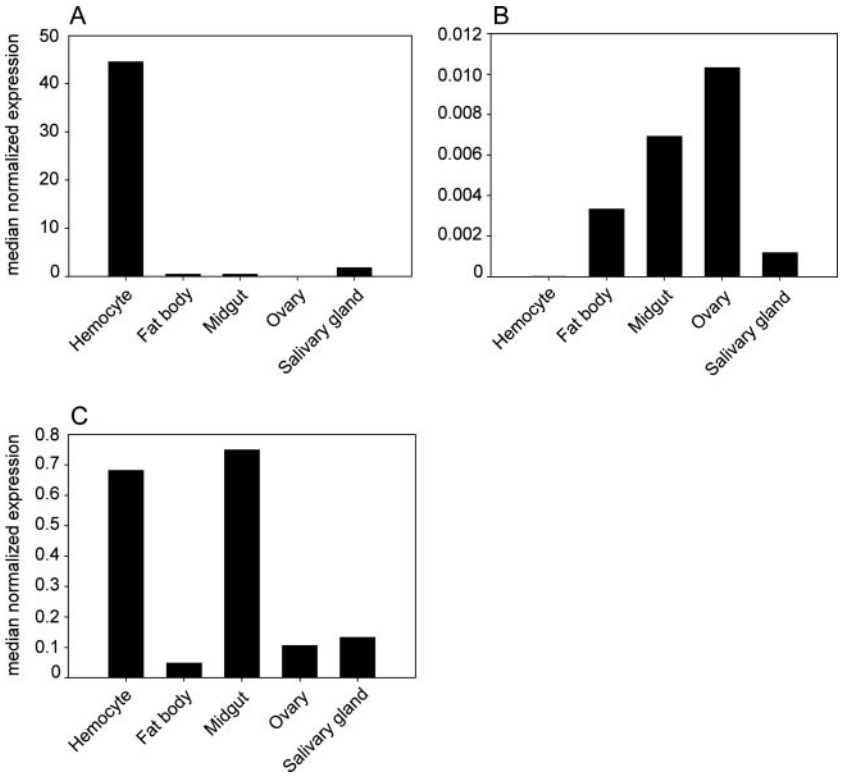

FIG. 3. Tissue distributions of defensin-1, defensin-2, and lysozyme. qRT-PCR was used to assess the basal-level expression (that in fed, uninfected ticks) of each antimicrobial in major tick tissues. (A) Defensin-1, (B) defensin-2, (C) lysozyme. The median normalized expression in each tissue, calculated as shown in equation 1, is reported.

fold; $P = 0.0074$) h suggested a lag effect on defensin-2 gene transcription in rickettsia-challenged ticks (Fig. 4D; Table 3).

(iii) Lysozyme in the midgut. Lysozyme tissue profiles support previous results described by Simser et al. (42); however, the expression of lysozyme in the midguts was almost as intense as that in hemocytes. We owe the differences between our results and the previous results for lysozyme distribution to the sensitivity of qRT-PCR (Fig. 4E; Table 3). Interestingly, a distinct negative difference in the levels of lysozyme gene expression in the fat bodies of the challenged and control groups occurred at 48 h (−28.12-fold; $P = 0.0008$) (Table 3).

DISCUSSION

Antimicrobial gene expression is the result of an undoubtedly complex detection/signaling pathway. The study of antimicrobial gene expression may provide clues to mechanisms employed by the tick's immune system to prevent the overgrowth of resident or imbibed microbes. Additionally, fluctuations in gene expression profiles may suggest rickettsial modulation of the host immune response as an evasion mechanism used by rickettsiae to gain access to their host. Ultimately, defining the balance between vector and pathogen will aid in

TABLE 2. Antimicrobial gene expression in response to feeding

Antimicrobial	Tissue	No. of replicates (control, challenged)[a]	Median difference (*n*-fold) (negative difference [*n*-fold])[b,d]	95% CI[c]	*P* value[d]
Defensin-1	Fat body	3, 3	+1.192	1.183–1.201	0.0893
	Midgut	3, 3	+35.34	35.09–35.60	5.812E−12
Defensin 2	Fat body	4, 5	+1.005	0.9770–1.034	0.9787
	Midgut	4, 4	+5.140	4.995–5.289	2.091E−06
Lysozyme	Fat body	3, 3	0.0585 (−17.09)	0.0087–0.3938	0.0138
	Midgut	3, 3	0.6047 (−1.654)	0.0899–4.070	0.0912

[a] Numbers of replicated experiments in which one tick served as a replicate.

[b] The difference (*n*-fold) is calculated as the ratio of normalized median values for challenged ticks to normalized median values for control ticks. Ratios of ≤1 are represented as −1/median difference (*n*-fold).

[c] 95% confidence intervals (95% CI) were calculated for the experimental median difference (*n*-fold) data by using the population variance derived as described by Price and Bonett (36) and Bonett and Price (1).

[d] Results for fed ticks were said to be significantly different from those for unfed ticks if the experimental median difference (*n*-fold) exceeded the upper or lower limit of the 95% confidence interval calculated for each antimicrobial population data set by using methods described by Price and Bonett (36) and Bonett and Price (1). Defensin-1 population critical limits, 0.9905 and 1.010; defensin-2 population critical limits, 0.8348 and 0.9001; lysozyme population critical limits, 0.0813 and 12.30. *P* values were derived from the antimicrobial population distributions as described in the appendix.

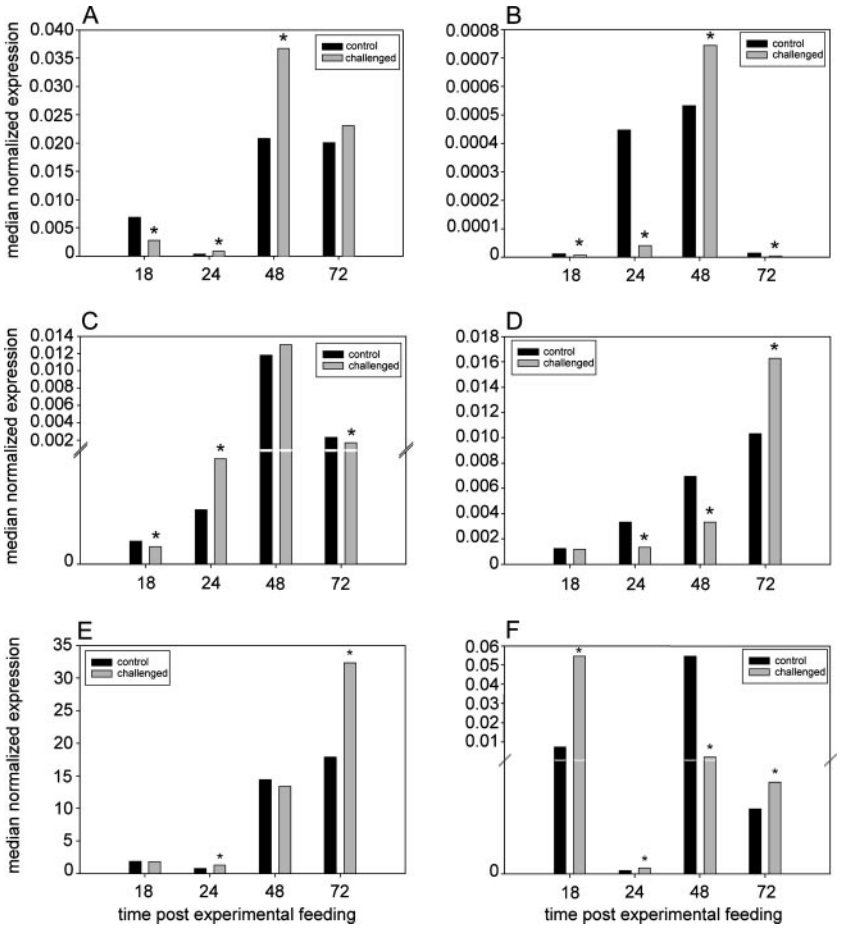

FIG. 4. Antimicrobial gene expression in response to challenge with *R. montanensis*. (A to E) Gene expression in the midguts. (B to F) Gene expression in the fat bodies. (A and B) Defensin-1 gene expression. (C and D) Defensin-2 gene expression. (E and F) Lysozyme gene expression. Four-day-old rabbit blood-fed female ticks were capillary fed blood spiked with *R. montanensis*-infected L929 cells. qRT-PCR was used to assess antimicrobial gene expression in response to *R. montanensis* challenge over time post-experimental feeding (shown in hours). Control ticks were capillary fed blood spiked with uninfected L929 cells. The median normalized expression for each time point, calculated as shown in equation 1, is plotted for both control and challenged ticks. Asterisks indicate a significant difference between challenge and control groups at the 5% level. See Table 3 for calculated differences (*n*-fold) and 95% experimental confidence intervals as well as the associated *P* values.

elucidating factors that determine symbiotic relationships as commensal or parasitic.

In this study, we report a new defensin isoform from *D. variabilis*, defensin-2, which belongs to the arthropod family of defensins. An alignment of defensin-2 with other tick defensins highlighted the conserved cysteines common to all arthropod defensins. Similarity to the other defensins was moderate but high enough to implicate defensin-2 as an antimicrobial. Recent reviews describe defensin as a multifunctional antimicrobial with abilities to act as signaling molecules that promote immune responses; as inhibitors of microbial nucleic acid, cell wall, and protein synthesis; and as pore-forming peptides (2, 14, 20). The variety in the functions of antimicrobials appears to be attributable, in part, to differences in primary structures and charges at physiological pHs (2, 20). The corresponding gene phylogeny (based on primary structures) and the predicted acidic pI of the mature defensin-2 peptide relative to those of other tick defensins, especially defensin-1, call into question the putative function of defensin-2. The observation that *Ornithodoros moubata* and *Amblyomma americanum* defensins group with one another and that defensin-1 groups with other Acari defensins may indicate that the gene phylogeny classifies similarly functioning peptides. These data argue for a difference in the antimicrobial specificity or the mechanism of antimicrobial action of defensin-2.

The potential for antimicrobial gene expression at the tissue level could feasibly determine the pervasiveness and intensity of microbial infection in the tick. Antimicrobial gene expression in ticks is localized to the hemolymph, hemocytes, midgut, and fat body (4, 11, 18, 23, 34, 35). In this study, we investigated the basal-level tissue distributions (those in fed, uninfected ticks) of defensin-1 and -2 and reexamined the lysozyme distribution using qRT-PCR. In the soft tick *Ornithodoros moubata*, there is a contrast in the tissue distribution patterns of defensin isoforms. *Ornithodoros moubata* isoforms A and B are expressed in the midgut exclusively; isoforms C and D exhibit a broader distribution, with expression of the corresponding genes occurring in the fat body and midgut (34, 35). In our study, defensin-2 exhibited widespread tissue distribution in comparison to defensin-1. The diffuse expression pattern of

TABLE 3. Antimicrobial expression in response to *R. montanensis* challenge

Antimicrobial	Tissue	Time (h)	No. of replicates (control, challenged)[a]	Median difference (*n*-fold) (negative difference [*n*-fold])[b,d]	95% CI[c]	*P* value[d]
Defensin-1	Fat body	18	4, 5	0.5731 (−1.74)	0.5336–0.6156	0.0041
		24	4, 4	0.0909 (−1.00)	0.0846–0.0976	0.0006
		48	4, 6	+1.396	1.230–1.499	0.0334
		72	4, 4	0.2131 (−4.693)	0.1984–0.2288	0.0008
	Midgut	18	7, 7	0.4001 (−2.5)	0.3885–0.4120	0.0022
		24	7, 7	+2.650	2.573–2.729	0.0001
		48	4, 5	+1.764	1.713–1.816	0.0016
		72	7, 7	+1.15	1.110–1.184	0.1842
Defensin-2	Fat body	18	4, 5	0.9504 (−1.502)	0.8530–1.059	0.5000
		24	4, 4	0.4045 (−2.472)	0.3630–0.4507	0.0060
		48	4, 5	0.4784 (−2.090)	0.4294–0.5331	0.0074
		72	4, 4	+1.579	1.418–1.760	0.0056
	Midgut	18	7, 8	0.7508 (−1.332)	0.7445–0.7573	0.0265
		24	7, 7	+1.932	1.916–1.948	0.0008
		48	4, 4	+1.102	1.093–1.111	0.1763
		72	6, 6	0.7297 (−1.37)	0.7235–0.7360	0.0265
Lysozyme	Fat body	18	4, 5	+7.576	6.975–8.229	1.386E−07
		24	3, 4	+1.868	1.720–2.029	0.0012
		48	4, 5	0.0356 (−28.12)	0.0327–0.0386	0.0008
		72	3, 4	+1.413	1.301–1.535	0.0099
	Midgut	18	4, 6	0.9725 (−1.028)	0.9182–1.030	0.1289
		24	6, 6	+1.636	1.545–1.733	0.0044
		48	4, 4	0.9302 (−1.075)	0.8784–0.9852	0.0758
		72	5, 6	+1.810	1.709–1.917	0.0017

[a] Numbers of replicated experiments in which one tick served as a replicate.
[b] The difference (*n*-fold) is calculated as the ratio of normalized median values for challenged ticks to normalized median values for control ticks. Ratios of ≤1 are represented as −1/median difference (*n*-fold).
[c] 95% confidence intervals (95% CI) were calculated for the experimental median difference (*n*-fold) data by using the population variance derived as described by Price and Bonett (36) and Bonett and Price (1).
[d] Results for challenged ticks were said to be significantly different from those for the controls if the experimental median difference (*n*-fold) exceeded the upper or lower limit of the 95% confidence interval calculated for each antimicrobial population data set by using methods described by Price and Bonett (36) and Bonett and Price (1). Defensin-1 fat-body population critical limits, 1.069 and 1.234; defensin-1 midgut population critical limits, 1.017 and 1.078; defensin-2 fat-body population critical limits, 0.6450 and 1.550; defensin-2 midgut population critical limits, 0.9965 and 1.014; lysozyme fat-body population critical limits. 0.9206 and 1.086; lysozyme midgut population critical limits, 1.030 and 1.155. *P* values were derived from the antimicrobial population distributions as described in the appendix.

defensin-2, similar to that of isoform C from *Ornithodoros moubata*, suggests tissue-specific roles for each of the defensins. Because *R. montanensis* infects the ovary and the sylvatic cycle is perpetuated through transovarial transmission, the abundant defensin-2 expression in the ovary is interesting. Future studies will address antimicrobial gene expression in the context of ovary invasion, survival of rickettsiae, and transovarial transmission. It is also of interest that our method of dissection of the fat bodies did not provide fat body cells free of tracheolar tissue. Because of this contamination, we cannot rule out the possibility that tracheolar tissue could be a source of defensin. However, we feel that this possibility does not detract from our findings given that the fat body is integral to defensin production in other arthropods.

As hematophagous arthropods feed, there is the potential to imbibe microbes that the host harbors. It is well documented that defensin is up-regulated in response to feeding in insects. In the blood-sucking fly *Stomoxys calcitrans*, the abundance of transcripts for defensins Smd 1 and 2 increases to peak levels at 24 and 36 h post-blood meal, respectively (25). A recent review discusses the immune signaling capabilities of cell-derived hydrophobic portions (hyppos) of molecules (41). It is possible that damage to blood cells, incurred as hemolysis progresses during blood meal digestion by the tick (30), could stimulate an immune response. Thus, antimicrobial gene expression during feeding may have evolved for dealing with the threat of invading microbes or for responding to free hydrophobic molecules in the midgut lumen. Therefore, the increase in the expression of defensin-1 and -2 genes in response to feeding was expected. Defensin-1 transcript levels in the fat bodies of both the fed and unfed ticks may have remained at constitutively high levels in "anticipation" of impending hemocoelic microbial infiltration.

Studies involving differential gene expression in *D. variabilis* in response to *R. montanensis* infection are not without precedent. Subtractive hybridization to decipher expression patterns in *R. montanensis*-infected and uninfected ovaries has revealed a number of ovary-specific, differentially expressed genes that were classified according to putative functions and include receptor and adhesion genes, genes for stress response pro-

teins, and most relevant to this study, immune function genes (28). Of equal interest was the use of a differential display to parse the same experimental design for ovary-specific differentially expressed genes. Macaluso et al. (26) found that Ena/vasodilator-stimulated phosphoprotein-like protein, vacuolar ATPase, and α-catenin transcripts were all up-regulated in infected ovaries, reflecting the potential importance of these proteins for rickettsial entry and intra- and intercellular mobility.

To date, there are no studies describing the rate at which or the intensity with which rickettsiae become established in the tick or the immune system recognition and action by the tick host. We assume that the recognition of rickettsiae by immune system effectors can occur as rickettsiae are acquired through blood feeding and as they begin their migration through the tick. As rickettsiae migrate from the gut to the hemocoel, they are exposed to soluble factors known (and unknown) to be active in the *D. variabilis* hemolymph (3, 4, 17–19). From our observations, we can say that rickettsiae effectively infect their tick host within 72 h postfeeding, as 100% of the ticks we tested at 72 h post-experimental feeding were infected with *R. montanensis*. One hundred percent detection of rickettsiae was not observed at earlier time points, possibly because the numbers of live bacteria were below the limits of detection for our assays. We chose to continue the analyses for the 18 (0%)-, 24 (50%)-, and 48 (50%)-h time points, as ticks were challenged with rickettsiae and visibly imbibed blood, which constitutes a bacterial challenge.

In general, we saw an increase in gene expression for all tested antimicrobials, as time increased, in both the midgut and fat body. In the midgut, we saw a positive difference between the challenged and control groups as early as 24 h and as late as 72 h post-experimental feeding. This finding may be correlated to the increase in the percentage of ticks with detectable levels of rickettsiae, i.e., the increase in the number of rickettsiae that had been imbibed at these time points. While the same general trend appeared to be true for the fat bodies, we noted an apparent delay in defensin-1 (18 to 48 h) and defensin-2 (24 to 48 h) gene expression at the time when rickettsiae may have been entering the hemocoel. Pathogen-directed modulation of antimicrobial gene expression is common. Down-regulation of β-defensin is observed when mice are infected with the obligate intracellular parasite *Cryptosporidium parvum* (48). Similarly, an inverse correlation between the *E. coli* multiplicity of infection and diptericin gene expression in the *Drosophila* cell line mbn-2 has been observed (15).

In summary, we have described a second defensin isoform, defensin-2, from the hard tick *D. variabilis*, a vector for the spotted fever group rickettsia *R. rickettsii*. The bioinformatic and phylogenetic analyses predict that defensin-2 has antimicrobial properties. The observation that defensin-2 is expressed in a number of tissues, especially the ovary, calls into question its specificity as an antimicrobial and its involvement in rickettsial infection of the ovary. Based on the gene expression data and statistical analysis, we accept our hypothesis that differential antimicrobial gene expression levels in the midgut and fat body occur in response to *R. montanensis* challenge. We are continuing our work with antimicrobials to answer questions regarding their functions as general antimicrobials and antirickettsial agents. Future work will address the importance of local versus systemic responses with regard to antimicrobial expression and defensin-2 gene expression in the ovary in response to rickettsial challenge. This is the first report of profiles of antimicrobial gene expression in a vector tick in response to a spotted fever group rickettsia acquired per os.

APPENDIX

qRT-PCR statistical calculation and notation. Due to the lack of known population parameters for each antimicrobial gene tested, a population corresponding to each antimicrobial was created to facilitate a tangible statistical assessment of the experimental values that represent FD. Our populations were derived from a pooled data set that was antimicrobial and tissue specific for the feeding experiments and antimicrobial specific for the *R. montanensis* challenge experiments. A decision regarding the pooling strategy was based on an assessment of the effect of the tissue origin with respect to antimicrobial gene expression.

For each antimicrobial gene, we combined values without regard for the experimental variables of time (rickettsia-challenged versus control group only) and treatment (rickettsia-challenged versus control group and fed versus unfed group) while leaving classifications based on tissue origins intact. We performed a Kruskal-Wallis analysis of variance with Bonferroni's correction for values corresponding to tissues at a 5% significance level.

We observed no effect from tissue origin on values from the feeding experiments; therefore, data from fed and unfed groups were pooled, i.e., an antimicrobial-specific data set was derived without regard for treatment (fed or unfed) or tissue origin. For *R. montanensis* challenge experiments, we observed a significant difference ($P < 0.0001$) between the midgut and fat-body expression patterns of each antimicrobial, indicating a tissue effect. Therefore, we decided to create antimicrobial-specific data sets comprised of all time points and treatments for individual tissues (i.e., defensin-1, fat body; defensin-1, midgut; and defensin-2, fat body, etc.). By pooling normalized expression values, the method accounts for both the biological variations (the ratio of the amount of blood imbibed/the dose received and the abilities of individual ticks to respond) and the technical variations (pipetting and assay precision), with the effects of the variations inherent to each replicate on normalized expression values being represented in the group.

Thus, for each tissue, the combined data sets were designated challenged′, control′, fed′, and unfed′. Next, to create the populations, a Monte Carlo simulation was used to resample, without replacement, the challenged′, control′, fed′, and unfed′ data sets and to calculate the median from each resampling for a total of 1,000 iterations. This resampling provided populations of median values for the challenged′, control′, fed′, and unfed′ data sets, now designated populations N_1 (challenged or fed) and N_2 (control or unfed). From N_1 and N_2, we calculated (i) the population median; (ii) the population variance, standard deviation, and standard error; and (iii) the replicate FD′ and population FD″. The population medians are represented by $\hat{n}_1$ and $\hat{n}_2$ for N_1 and N_2, respectively. The variances in the populations were calculated according to previously published methods (1, 36) as shown below in equation A1:

$$\mathrm{var}N_1 = \{[\ln(Y_{(n-a+1)}) - \ln(Y_{(a)})]/2z\}^2 \quad \text{(A1)}$$

where $\mathrm{var}N_j$ is the variance in population N_j, Y represents independent ordered and continuous random variables, n is 1,000, a is 463, and z (the critical value on the standard normal distribution) is 2. The standard deviation of the population values was calculated using methods described by Price and Bonett (36) and Bonett and Price (1), and the formula is given as equation A2:

$$\sigma_{\mathrm{median}} = \exp[(\mathrm{var}N_1 + \mathrm{var}N_2)^{1/2}] \quad \text{(A2)}$$

The standard error of the median ($\mathrm{SE}_{\mathrm{median}}$) was calculated as described previously (24) as shown by equation A3:

$$\mathrm{SE}_{\mathrm{median}} = 1.253(\sigma_{\mathrm{median}}/\sqrt{n}) \quad \text{(A3)}$$

where 1.253 accounts for the 25% inflation observed in the median compared to the mean (24) and n is 1,000. FD′ was calculated as $(n_{1,1}/n_{2,1})$, $(n_{1,2}/n_{2,2})$, . . . , $(n_{ji},/n_{ji},)$ for each of the 1, 2, . . . , i resamplings for groups 1 (challenged or fed) and 2 (control or unfed). FD″ was calculated as $\hat{n}_1/\hat{n}_2$, representing the population FD.

The 95% confidence interval was calculated for both the experimental data and the population data. The experimental confidence interval was calculated according to Price and Bonett (36) and Bonett and Price (1) as shown below by using the population variance:

$$\left(\frac{n_1}{n_2}\right)\exp[\pm 1.96(\mathrm{var}N_1 + \mathrm{var}N_2)^{1/2}] \quad \text{(Aa)}$$

The population confidence interval was calculated according to Price and Bonett (36) and Bonett and Price (1) as shown below.

$$\left(\frac{\hat{n}_1}{\hat{n}_2}\right)\exp[\pm 1.96(\mathrm{var}N_1 + \mathrm{var}N_2)^{1/2}] \quad \text{(Ab)}$$

A table of cumulative probabilities of the standard normal deviate for each antimicrobial peptide population was calculated so that P values could be derived. To calculate cumulative probabilities, four values were required: (i) the absolute deviation of FD′ from FD″, (ii) $\ln(Z')$ where Z' is the population Z value, (iii) the normal cumulative distribution Φ for $\ln(Z')$, and (iv) $(1 - \Phi)$. All values are defined below.

(i) The absolute deviation is given by $\mathrm{FD}'_1 - \mathrm{FD}''$, $\mathrm{FD}'_2 - \mathrm{FD}''$, . . . , $\mathrm{FD}'_n - \mathrm{FD}''$.

(ii) The natural log of Z' was determined to gain sensitivity in the tails of the distribution of Z′ values and is given by

$$\ln(Z'_1), \ln(Z'_2), \ldots, \ln(Z'_n) = \ln\left(\frac{|\mathrm{FD}'_1 - \mathrm{FD}''|}{\mathrm{SE}_{\mathrm{median}}}\right),$$

$$\ln\left(\frac{|\mathrm{FD}'_2 - \mathrm{FD}''|}{\mathrm{SE}_{\mathrm{median}}}\right), \ln\left(\frac{|\mathrm{FD}'_n - \mathrm{FD}''|}{\mathrm{SE}_{\mathrm{median}}}\right) \quad \text{(Ac)}$$

(iii) The normal cumulative distribution (Φ) is given by NORMSDIST($\ln(Z'_1)$), NORMSDIST($\ln(Z'_2)$), . . . , NORMSDIS T($\ln(Z'_n)$) by using Excel's normal cumulative distribution function.

(iv) For probabilities greater than or equal to each calculated Z' value in step iii, we simply calculated $(1 - \Phi)$.

To calculate P values, we performed a Z transformation of each experimental FD and noted $P \geq Z'$ from our antimicrobial gene-specific cumulative probabilities table. Our Z transformation of the experimental FD values was calculated using equation A4 to derive P values.

$$Z' = \ln\left(\frac{|\mathrm{FD} - \mathrm{FD}''|}{\mathrm{SE}_{\mathrm{median}}}\right) \quad \text{(A4)}$$

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