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SHORT COMMUNICATION

Not all spider silks are antimicrobial

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Abstract. Spider silks are thought to have antimicrobial properties that prevent rapid degradation. Despite its biomedical potential, little research has focused on antimicrobial properties of spider silks. We tested the antimicrobial properties of gluey gumfoot capture threads of the western black widow spider, *Latrodectus hesperus* Chamberlin & Ivie, 1935, both for whole threads and after separating viscid silk from water soluble glue components using a water wash. Capture threads, unlike dry dragline silk, have a glue coating that may promote different interactions of bacteria with the material. Antimicrobial activity was assessed in an aqueous *Escherichia coli* cell culture and growth of colonies was counted over 24 hours. In contrast with previous research, mostly on dry dragline silk, we found an increase in bacterial growth for whole gumfoot thread treatment, but no effect of the solid and water-soluble components individually. These results suggest that better and proper procedures are needed for testing antimicrobials in silks, with controlled experiments using just one type of silk.

Keywords: Antimicrobial properties, black widow, viscid silk, major ampullate, aggregate glue

Spiders are a diverse group, with approximately 48,000 species described to date (World Spider Catalog 2019) and a nearly 400 million year history (Shear et al. 1984). All spiders make silk throughout their lives, and it is essential to their survival. Spiders produce up to seven different types of chemically and physically distinct silks spun from discrete glands and spigots, which are used for a great variety of functions (e.g., egg sacks, prey-wrapping, web-building). Each type of silk protein varies in amino acid composition and molecular structure (Vollrath 1992), which can ultimately determine their properties. While silk mechanical properties are increasingly well understood (e.g., Moore & Tran 1999; Lawrence et al. 2004; Blackledge et al. 2005) much is still unknown, including silk's biomedical potential. Spider webs were used by Greeks and Romans to heal battle wounds and stop bleeding, and as a cure for sore throats (Laguna 1955; Lewis 1996; Benítez 2011).

In nature, different types of silks are required to function for different periods of time. For example, silk in orb webs is typically recycled on a daily basis, as spiders remove and consume the old silk and then spin a new orb (e.g., Breed et al. 1964). Other spiders, such as cobweb weavers, spin webs with backbones of dragline silk that may persist longer, up to several days or weeks (Janetos 1982). Furthermore, silk can improve its mechanical properties as it ages. Dragline silk kept in the lab shows improvement in its mechanical performance during the first year of aging (Agnarsson et al. 2008). Signs of degradation, indicated by a decrease in toughness, elasticity and breaking load, do not start to appear until four years after harvesting (Agnarsson et al. 2008). This observation, along with the long-term persistence of silk in the environment and anecdotal accounts of its use as "antiseptic" wound dressings (Laguna 1955; Benítez 2011), has led to the view that spider silks have antimicrobial properties to prevent rapid degradation.

Antimicrobial activity may be caused by biochemical inhibitory agents (Neu 1992) or by physical characteristics of material surfaces (Ivanova et al. 2012); each mechanism may either kill microbes (bactericidal) or inhibit their reproduction (bacteriostatic) (Pogodin et al. 2013). Several studies have looked at antimicrobial properties of spider silk and found varied support for bacterial growth inhibition (Table 1), but none have successfully identified a mechanism of antimicrobial activity. A few studies suggest that silk is a bacteriostatic rather than bactericidal inhibitor (Roozbahani et al. 2014), possibly due to physical properties of the silk surface rather than chemical properties (Sharma 2014; Babczyńska et al. 2019). Testing silks' antimicrobial properties is challenging and only a few of these studies conducted controled experiments with proper use of the same kinds of silks (Andersen 1970; Amaley et al. 2014; Babczyńska et al. 2019). This is critical for reliable results, as different spider silks are chemically diverse, and in a single web (e.g., cobweb, sheet web, orb web) spiders use different types, both fibrous and glues (Vollrath 1992), which may account for some of the contradictory results seen in previous research. Moreover, despite the diversity of silk types and microorganisms, only a very few have been tested.

Here we examined the gumfoot silk from the western black widow, *Latrodectus hesperus* Chamberlin & Ivie, 1935 to determine if it has antimicrobial properties against Gram negative bacteria, using *Escherichia coli* as a representative organism. Black widows build a semi-permanent three-dimensional web that consists of a supporting structure and vertical lines under tension, termed gumfoot threads, which connect to the substrate (Fig.1) (Benjamin & Zschokke 2002). Gumfoot silk consists of dragline silk coated with aggregate glue droplets that facilitate prey adhesion. Dry dragline silk is made mainly of glycine, alanine and proline (Lawrence et al. 2004) while aggregate glue is made of glycoproteins (Vollrath & Tillinghast 1991) and an aqueous solution of low molecular mass compounds (LMMC) (Vollrath et al. 1990). Thus, compared to the more commonly investigated and much larger dragline silk proteins, aggregate glue may provide a very different substrate for bacterial growth. *Escherichia coli*, for example, can utilize many different carbon and nitrogen sources to generate many possible combinations of nutrients. Therefore the presence of small molecules such as choline, gamma-aminobutyric acid and sugars (Jain et al. 2015) in gumfoot threads from *L. hesperus* viscid silk could have implications for how microbes interact with silk.

One hundred gumfoot silk strands (5 mm length each) were collected on a sterilized glass fork from about five webs of sexually mature female *L. hesperus* per replicate. Gumfoot silk components were tested both together, using whole unwashed threads, and on their own, by washing the threads with 5 mL of sterile deionized water to separate the aqueous LMMC from the dry dragline silk. Because glycoproteins are not water soluble (Amarpuri et al. 2015), glycoproteins remained in the silk, and just the low molecular mass compounds are present in the washed residue. The unwashed silk ($n = 3$), washed residue ($n = 3$), and dragline silk plus glycoproteins ($n = 3$)

Table 1.—Summary of research on antimicrobial properties of spider silk up to date.

Spider	Silk	Sample Preparation	Experimental Method
Unknown	Web	Unknown	Petrifilm in a solution of distilled water and the bacteria
Crossopriza lyoni (Blackwall, 1867)	Cobweb	One mg/mL protein solution of spider silk dissolved in hydrochloric acid and acetic acid (50:50 v:v) and neutralized with sodium hydroxide to separate the proteins.	Minimal Inhibitory Concentration (MIC) after serial dilution of the protein in liquid Mueller Hinton broth in micro dilution plates and measured of absorbance at 620 nm using a Micronaut system
Tegenaria domestica (Clerck, 1757)	Sheet web	Liquid broth incubation of native silk, silk exposed to UV, soaked in DI water and Proteinase K.	Light absorbance reading using Photo-spectrometer
Unknown	Web	Silk extracts using different solvents to (methanol, ethanol, acetone and distilled water)	Disc diffusion assay
Pholcus phalangioides (Fuesslin, 1775)	Cobweb	Webs (500 mg) in a 5 mL solution of distilled water 1% Tween80 and 5% acetone	1. Kirby-Bauer test: well diffusion method for 24, 48 and 72 hours. 2. Minimum inhibition concentration (MIC) of solution were using Macro Broth Dilution method
Nephila pilipes (Fabricius, 1793)	Dragline silk (**Not confident it is exclusively dragline)	Dragline silk threads (~ 1 mg) gently washed in sterilized distilled water.	Antimicrobial assay was performed using agar plates.
Argiope aurantia Lucas, 1833	Reeled dragline silk	Organic extracts (70% Methanol, 70% ethanol, chloroform) of silk and native dry dragline silk	1. Spot assay to examine the presence of any non-diffusible inhibitory agent. 2. Bacterial growth curve using AATCC 100 Assay (Assessment of antibacterial activity) 3. Scanning Electron Microscopy analysis- to investigate whether bacteria adheres to spider silk
Stegodyphus dumicola Pocock, 1898	Non-sticky retreat silk and capture cribellate silk from webs	Native non-sticky retreat silk, native capture cribellate silk, 0.5 mL ethyl acetate non-sticky retreat silk solution and 0.5 mL ethyl acetate capture cribellate silk	Bacterial growth inhibition assay.
Pholcus phalangioides	Web	Three hundred mg of silk dissolved in 30 ml of 5% NaOH, used as 100% and 50% concentration.	Kirby-Bauer Disk Diffusion Susceptibility Analysis Protocol
Cyclosa confraga (Thorell, 1892)	Orb web	Fifty mg dissolved in 100 ml 2.5% sodium hydroxide solution, used in solutions of different concentration (100%, 75% and 50%)	Kirby-Bauer Disk Diffusion Susceptibility Analysis Protocol
Parasteatoda tepidariorum (C.L. Koch, 1841)	Egg sac	The eggs and empty egg sacs	Incubated on lysogeny (LB) agar medium on petri dishes to observe potential bacterial presence.
Pardosa brevivulva Tanaka, 1975	Web	One mg of silk dissolved in 10 mL of the following solvents: chloroform, formic acid, ethanol, methanol, water and 1 N HCL. Dialysis of silk extracts, re-dissolved in dimethyl sulfoxide (DMSO fraction of silk)	1. Antibacterial: Disk diffusion method using silk extracts and DMSO fraction of silk. 2. Antifungal: Agar well diffusion method using silk extracts and DMSO fraction of silk. 3. Coomassie Brilliant Blue dye using bovine serum albumin as the standard. 4. FT-IR, ^{13}C & ^{1}H NMR and C_{18} column RP-HPLC analysis for characterization of antimicrobial compounds.

Table 1.—Extended.

Microorganism (-Gram negative/ +Gram positive/Fungi)	Findings	Reliability	Reference/ Publication Type
−*Pseudomonas fluorescens*	Inconclusive	No evidence	(Borders 2001)/Bulletin report
−*Escherichia coli* −*Pseudomonas aeruginosa* +*Staphylococcus aureus* +*Enterococcus faecalis* −wild strains of bacteria like MRSA, MBL-positive	MIC values of all Gram negative organisms including MBL producing strains were much lower (<10 mcg/mL) than that of the Gram positive organisms (>30 mcg/mL).	Some evidence	(Chakraborty & Das 2009)/Abstract
−*E. coli* +*Bacillus subtilis*	Native silk inhibited the growth of Gram positive for a short period of time, thus the active agent potentially acts in a bacteriostatic rather than bactericidal manner. No inhibition was detected against the Gram negative bacterium. Treatment of the silk with Proteinase K appears to reduce the ability to inhibit bacterial growth.	Strong evidence	(Wright & Goodacre 2012)/Journal Article
−*E. coli* +*B.* subtilis	Webs showed higher inhibition zone for Gram positive compared to Gram negative bacteria, with the acetone silk extract, which have the least polarity, showing the most antibacterial activity.	Weak evidence	(Mirghani et al. 2012)/ Conference Paper
−*E. coli* +*Listeria monocytogenes*	Spider silk solution inhibit the growth of bacteria, having a higher inhibitory effect against Gram positive than Gram negative bacteria.	Weak evidence	(Roozbahani et al. 2014)/Journal article
−*E. coli* −*P. aeruginosa* −*Klebsiella pneumoniae* +*S. aureus*	Growth was inhibit for all bacteria tested except *K. pneumoniae*. This strain of bacteria grew up to the margin of silk but not on silk threads. **Same results were observed in one year old silk.**	Weak evidence	(Amaley et al. 2014)/ Journal Article
−*E. coli* −*P. aeruginosa* +*B. subtilis*	Organic extracts of spider silk in spot analysis showed absence of any non-diffusible agent. Microbial growth curves showed no significant effect on bacterial growth patterns. SEM showed low adherence of Gram negative bacteria onto the silk surface Spider silk did not show resistance of adherence of Gram positive bacterium.	Strong evidence	(Sharma 2014)/Master Thesis
+*Bacillus thuringiensis*	Native spider silk from both the capture web and retreat significantly inhibited the growth while solutions did not differ from the control.	Strong evidence	(Keiser et al. 2015)/ Journal Article
−*Acinetobacter baumannii* −*Pasteurella multocida* +*S. aureus* +*Streptococcus pneumoniae*	Spider silk solution inhibit the growth of bacteria even at 50% concentrations.	Weak evidence	(Tahir et al. 2017)/ Journal Article
−*Acinetobacter* sp. +*Streptococcus* sp.	Spider silk solution inhibit the growth of bacteria at all three concentrations, showing higher inhibition for Gram negative than Gram positive bacteria.	Weak evidence	(Tahir et al. 2018)/ Journal Article
N/A	While outer surface of cocoons may have presence of microorganism, in the samples of eggs, no growth of bacteria was detected. This indicated that the eggs inside cocoons were sterile.	Strong evidence	(Babczyńska et al. 2019)/Journal Article
−*Klebsiella pneumoniae* −*Pseudomonas aeruginosa* −*Proteus vulgaris* −*Salmonella typhi* +*Bacillus megaterium* +*Staphylococcus aureus* Fungi: *Aspergillus niger* Fungi: *Aspergillus flavus* Fungi: *Candida albicans* Fungi: *Ustilago maydis* Fungi: *Altenaria solani* Fungi: *Mucor hiemalis*	The silk extract in acid formic (10% *w* / *v*) was able to inhibit the growth of *B. megaterium, S. typhi, K. pneumoniae, A. flavus, C. albicans, U. maydis*, and *A. solani*. FT-IR spectrum of DMSO fraction of the silk revealed the presence of hydroxyl group, alkyl group, alkenes group, amidic group, and sp2-hybridized C-H bonds.	Good evidence	(Phartale et al. 2019)/ Journal Article

Table 1.—Continued.

Spider	Silk	Sample Preparation	Experimental Method
Nephila pilipes (Fabricius, 1793) *Hippasa holmerae* Thorell, 1895 *Cyrtophora moluccensis* (Doleschall, 1857)	Orb web, funnel web and tent web respectively	1. Bundled spider silk (radial silk plus spiral silk) 2. Thin layer of portions of freshly built webs mounted on a 9 mm ring	1. Cross-streaking assays 2. Bacteria cultured directly on spider silk using three different nutrient level media: nitrogen-free glucose (nutrients without nitrogen), phosphate-buffered saline (no nutrients) and Luria-Bertani broth (full nutrients, including nitrogen)

were then tested for antimicrobial properties in an aqueous cell culture. An overnight culture of *E. coli* was prepared and aliquoted into 5 mL volumes for treatment with silk and residue. Unwashed silk and dragline silk were added to the culture on sterile forks, while washed residue was added as a suspension in 5 mL of deionized water. Each type of silk was inoculated into three culture volumes, and triplicate cultures with a sterile fork minus silk were prepared as a control. Cultures were incubated at 37°C for 24 hours. Growth of the cultures was assessed at 0, 6, 12 and 24 hours. At each time step, cultures were inoculated in triplicate onto 50% trypticase soy agar and incubated at room temperature; then colony-forming units per mL of culture (CFUs/mL) were counted the following day. A two-way repeated measure ANOVA was used to determine if the number of CFUs/mL differed among our four groups over time.

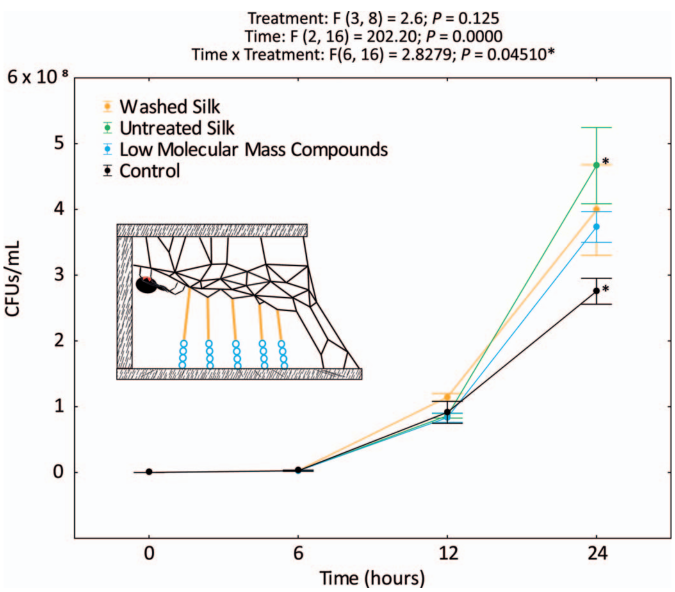

Figure 1.—Microbial growth curve of *E. coli* for four groups over 24 hours: untreated silk (green), washed silk (yellow), low molecular mass compounds (blue) and no-silk control (black). The *P-value* threshold of significance for our factorial ANOVA was 0.05. Error bars represent standard error of the mean. While treatment itself didn't show any effect ($P = 0.125$), time did ($P = 0.0000$) as the number of bacterial colonies for all treatments increased over time. The interaction between treatments and incubation time showed a significant difference between the control and the untreated silk at 24 hours ($P = 0.04510$, *post hoc* $P = 0.0031$).

Our study showed an increase in CFUs/mL for untreated gumfoot silk at 24 hours (Fig. 1). Neither the aqueous components nor the washed dragline silk showed any difference in *E. coli* growth compared to the control. These results suggest that, counter to the hypothesis that spider silk has anti-microbial properties, gumfoot silk from black widows may facilitate bacterial growth of Gram negative bacteria, but only in the presence of both dragline axial threads and aggregate glue. When small molecules are removed from the capture silk system, glycoproteins collapse, causing silk to lose its adhesive properties (Sahni et al. 2014), which may also affect its interactions with bacteria. We are unsure if our concentration of material is enough to have a significant effect; by washing the silk we reduce the concentration of both components, so an alternative hypothesis is that these treatments may simply not provide enough material to show the same effect on bacterial growth seen for whole threads. Future studies should look at different concentrations of small molecular mass compounds and glycoproteins to decouple the concentration from chemical changes as the cause of differences in antimicrobial activity profiles between the groups. Visualization of samples could also be vital to see differences in cultures and look at how the physical properties are enabling an increase in bacteria. Although cobwebs are long-lasting, gumfoot silk may not need to be resistant to bacterial degradation because black widows can easily repair or replace the capture threads without affecting the structural integrity of the web.

In the face of increasing global resistance to antimicrobials (e.g., CDC 2019; O'Neill 2016), novel strategies to prevent infection need to be explored. One such strategy is to investigate materials that have purported antimicrobial properties. Here we have expanded the range of spider silks whose antimicrobial properties have been studied. Our results demonstrate that it may be flawed to assume that all spider silks are antimicrobial. Silks from a broader range of spider species should therefore be studied to determine how widespread antimicrobial properties may be and how they may relate to phylogeny and ecology.

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Table 1.—Extended. Continued.

Microorganism (-Gram negative/ +Gram positive/Fungi)	Findings	Reliability	Reference/ Publication Type
+ *B. subtilis* + *Bacillus altitudinis* (Isolated bacteria from detritus and decorations on the webs of *Cyclosa mulmeinensis* (Thorell, 1887)) − *Enterobacter bugandensis* (Isolated bacteria from web of *N. pilipes*) − *E. coli*	Spider silk does not show anti-bacterial properties but prevents bacterial growth by limiting nitrogen accessibility concluded from the following results: 1. Spider silk, in its native state, does not inhibit the growth of bacteria even when in direct contact 2. Bacteria can grow on spider silk when supplemented with a full complement of nutrients 3. Bacteria grow poorly, or not at all, if nitrogen is withheld, even among an otherwise full complement of nutrients	Strong evidence	(Zhang et al. 2019)/ Journal Article

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