The antimicrobial properties of spider silk

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Thesis submitted to the University of Nottingham

for the degree of Master of Research.

February 2011

Abstract

The natural world is the source of many therapeutic products. Spider silk was evaluated for its ability to inhibit the growth of bacteria. Additionally tests assessing its potential suitability for use in human medicine were carried out.

Silk produced by certain species of spiders has the ability to inhibit the growth of a gram-positive bacterium, *B. subtilis*. The ability of the silk to inhibit bacteria does not appear to be common to all spiders. Of all the species of spiders examined in this study, only the web silk of *Tegenaria domestica* and the egg silk of *Pityohyphantes phrygianus* was shown to significantly inhibit the grow of bacteria. With the *T. domestica* silk it appears that the antimicrobial effect is short lived, the growth of bacteria was only inhibited after 24 hours of growth, but not 48 hours. The *P. phrygianus* egg silk it did not appear to shown a reduction in its antimicrobial properties over time, inhibition of bacteria was observed equally at 24 hours and 72 hours. There was no evidence to suggest *T. domestica* silk was also able to inhibit the growth of fungi. While there was a trend of silk from the spiders of the genera *Zilla* and a linyphiid to inhibit the growth of bacteria, it was not significant. Two other genera of spider, *Araneus,* and *Lasiodora* did not appear to possess antimicrobial silk.

Treatments carried out on the silk appear to show the antimicrobial property is not lost after being subject to UV light, but that after being treated with Proteinase K or heated to 80°C the silk was no longer antimicrobial, indicating that the antimicrobial compounds may be proteins.

Tegenaria silk was examined for its effects on the growth of mammal cells. The spider silk did not appear to inhibit the growth of mammal cells suggesting there is potential application in medicine.

Atypus silk was examined for its suitability as a bandage. It was found to be highly water proof but experiments showed high levels of bacteria present which would make *Atypus* silk unsuitable as a bandage.

Acknowledgements

First and foremost I'd like to thank my supervisor, Dr Sara Goodacre for all her help in making my year of research such an enjoyable one. Whether it was her endless supply of ideas or her unparalleled ability to make me feel positive about my work her contribution to this project was invaluable.

My family's support was a great help, their emotional, financial and intellectual assistance made the year far more enjoyable and productive. Their interest and pride in my research inspired me no end.

Working in such a friendly and helpful lab as the spider lab benefitted me immensely, Melanie's great sense of humour ensured that working in the lab was always a pleasure and her brilliantly inquisitive mind could be relied upon to assist me with my research. Jon's encyclopaedic knowledge of spiders and generosity in getting me supplies, either spiders or equipment, helped me greatly in this year. Dave Wragg also deserves a special thank you for very kindly personally collecting masses of spiders used in my research.

I'd also like to thank Kelly Ann-Vere for letting me do experiments with mammal cells. More than that she was endlessly generous with her time helping me use the equipment and setting up experiments.

Finally I'd like to thank everyone who attended our lab meetings to provide assistance and ideas; Professor J Brookfield, Professor B Clark, Dr M Liu, P Richards, S Boardman and N Lowe.

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Introduction

1.1.1. History of Spiders

The oldest true spiders appeared around 380-374 million years ago (Selden 1990) and the oldest known species is *Attercopus fimbriamguis*. After that there is fossil evidence of spiders belonging to the *Tetragnathidae* and *Deinopcdies* in the early Cretaceous period (Selden, Shear et al. 1991). Our understanding of the evolutionary origins of spiders is incomplete (Coddington and Levi 1991) although there is good fossil evidence to suggest that the *Araneae* originated in the late Silurian (420mya) period, and that there was major radiation of *Anareomorphae* during the late Palaeozoic (250mya) or early Mesozoic periods (Coddington and Levi 1991), see Figure 1.

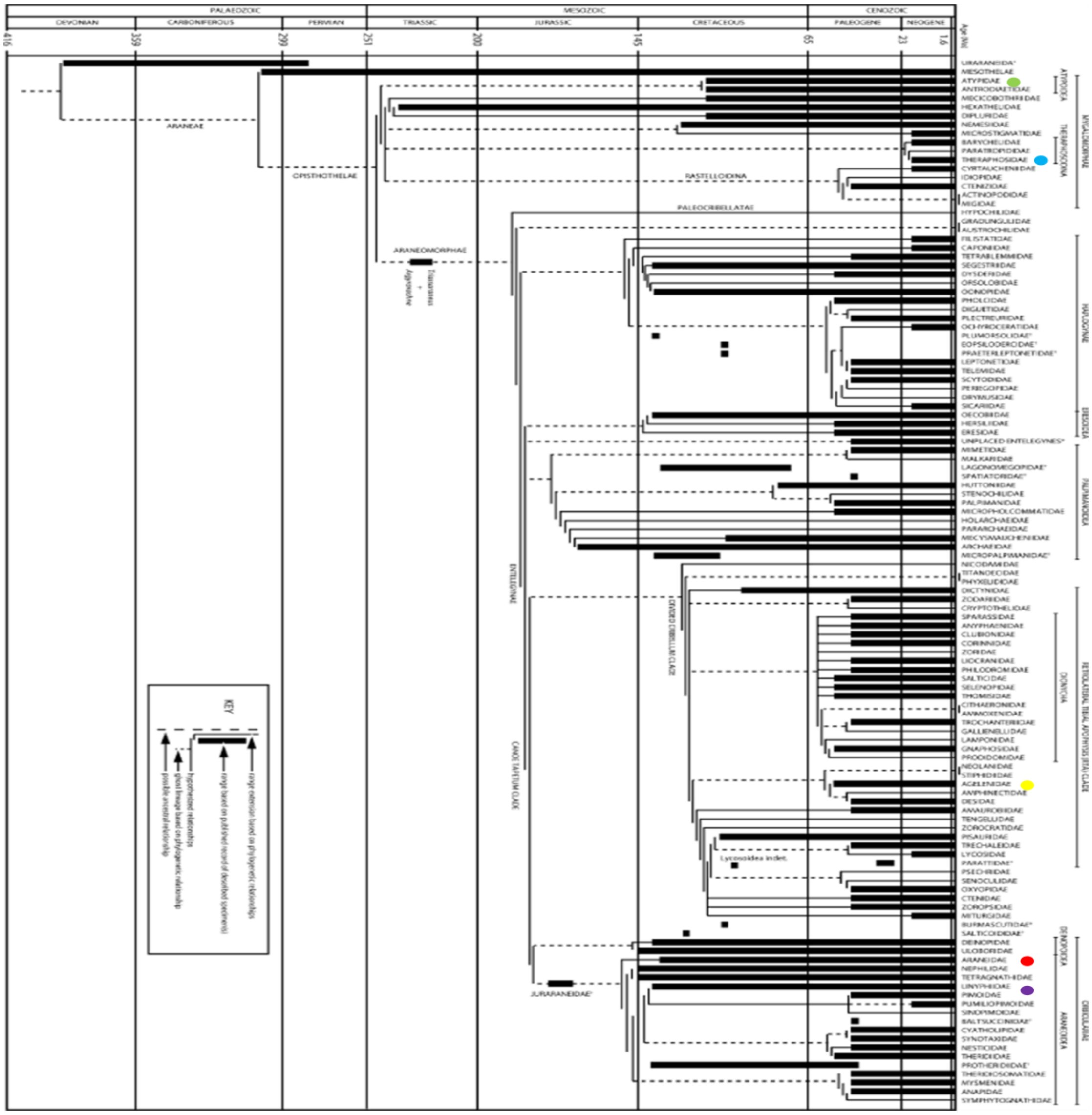


Figure 1: Evolutionary tree showing.... taken from(Selden and Penney 2010)

The coloured circles indicate where the spiders gathered for this project are on the evolutionary tree.

= *Atypus* species

= *Lasiodora parahybana*

= *Tegenaria domestica and Tegenaria duellica*

= *Araneus diadematus and Zilla diodia*

= *Pityohyphantes phrygianus* and other linyphiids

Spiders were originally classified as belonging to one of two groups on the basis of their silk spinning apparatus. These two groups are ecribellate spiders, which include the Mygalomorphae and Mesothelae, and cribellate spiders which include the Anareomorphae. The cribellium is a silk spinning organ found on cribellate spiders. The Myglomorphs and the Mesothelae lack a cribellium and have a anterior medium spinneret instead (Coddington and Levi 1991). However it has subsequently been discovered (Lehtinen 1967) that many anareomorphae spiders including *A. diamatus* also lack a cribellium. Coddington and Levi (1991) hypothesise that the ancestor of the Araneae was cribellate. Cribellate silk is very fine silk, of nanometre diameter (Vollrath 2000). The very fine threads are combed into hacked bands which provide elasticity and adhesion (Vollrath 2000). Ecribellate spiders coat the axial threads with an aqueous solution which forms sticky droplets, to provide the elasticity and adhesion (Vollrath 2000).

1.2.1. Spider morphology

Spiders are a diverse collection of animals, taxonomically grouped within the order Araneae, which in total contains more than 40,000 species across 109 families (Platnick 2010). The Araneae order is split into three distinct sub-orders, the Mesothelae, which contains around 350 species, the Mygalomorphae which compromise approximately 1,500 species, and the Anareomorphae which contain the remaining 90% of spider species (Foelix 1996). While diverse in size and morphology, all spiders have particular anatomical features in common. These are two body segments, called the abdomen and the cephalothorax, which are joined by a narrow stalk called a pedicel (Roberts 1995). Spiders also universally posses eight legs (provided they are healthy) either six or eight eyes and spinnerets for extruding silk. The parts of spider morphology that are of most interest to this project are the silk glands and the spinnerets, where the silk is manufactured and

released respectively. The silk is produced in silk glands which lie inside the abdomen, and then extruded through openings called spigots, which lie on the spinnerets.

The spinnerets are located at the hind end of the abdomen split in three pairs, the posterior, the anterior and the median spinnerets (Roberts 1995). It is the procedure of how the silk is combined in the spigots that gives spider silk its unique and hard-to-replicate properties (Scheibel 2004). There is a variety of spinneret forms across the Araneae order (Marples 1967). There are four areas where spinnerets are on spiders, the anterior median, the anterior lateral, the posterior median and the posterior lateral (Marples 1967). However different groups of spiders have differing arrangements and not all spiders have all four spinnerets. Additionally Yoshida (1999) observed that Theridiidae have huge aggregate spigots and noted that there is a relationship between a spider's predatory behaviour and both the number of Aciniform spigots and the size of aggregate spigots. Coddington (1989) describes the Araneidae arrangement as conservative and consistent. In Araneidae the cuticle sculpturing is lenticular or squamate as opposed to fluted or grooved (Coddington 1989). In Araneidae the piriform spinning field is uniform across the anterior lateral spigot (Coddington 1989). There are three classes of spigots on the posterior median spinneret in Araneidae; the anterior cylindrical spigot, the single posterior minor ampullate spigot, and many small Aciniform spigots (Coddington 1989). In Araneidae the posterior lateral spinneret is the most complex, compromising a mesal basal margin of two cylindrical spigots, a anterolateral margin of flagelliform spigots and two aggregate glue gland spigots (Coddington 1989). Additionally, across the posterior lateral spinneret is the second group of Aciniform spigots (Coddington 1989).

The cephalothorax is the anterior segment of the spider and it is on this that the spiders legs, palps, mouthparts, eyes and chelicerae are located (Roberts 1995).

Certain internal parts, the central nervous system, the poison glands and the stomach are contained in the cephalothorax (Roberts 1995). Of these, the spider's legs have most contact with silk most as the spider walks across their webs. Additionally the spider's mouth regularly comes into contact with silk. Many species have regular mouth contact with the silk as some species of spider wrap their prey in tubiliform silk before eating the prey and orb-web spiders will eat their webs daily.

There are some common features to the appendages of the cephalothorax. The chelicerae, located in front of and above the spider's mouth, are commonly the spider's primary form of attacking and subduing prey. Most frequently these are used to either bite prey, inject prey with venom or sometimes simply incapacitate prey by crushing (Roberts 1995). The chelicerae are also likely to come into regular contact with the spider's silk as many of the spider's prey are in the silk webs before being incapacitated by the chelicerae. Behind the chelicerae are the palps, which while possessed by both male and female spiders, become greatly modified in sexually mature males. Palp morphology is the primary method by which males are identified to species level (Roberts 1995). The primary function of the palps is for male sperm transfer (Roberts 1995). The sperm on the male palps is delivered in a sperm web, manufactured from silk. Behind the palps there are 4 legs on each side, and each of the eight legs is split into 7 segments; the coxa, the trochanter, the femur, the patella, the libra, the metatarsus and the tarus. It is on the tarus that the claws are located, while there is variation across the Araneae order as to number of claws, but for the web spinning spiders there are three claws on each tarus (Roberts 1995). The extra claw is used to grab the web as the spider crosses it and is a possible mechanism by which spiders avoid getting stuck to their own webs.

The Araneae is an almost entirely carnivorous group, which feeds mainly on insects, but also substantially on other arthropods and other spiders. While several specialist feeders exist, spiders tend to be generalist feeders (Wise 1993). Vollrath (1999) states that there is considerable variation in the mechanical properties of silk produced under different environment conditions, even by the same individual (Madsen, Shao et al. 1999). Studying amino acid composition of dragline silk across a range of Araneidae spiders Work and Young (1987) found that intraspecific variability was larger than interspecific variability. This indicates that selection on dragline silk acts to optimise a compromise silk that functions well under a wide range on conditions. This means that silk is more of a generalist, rather than specialist material (Vollrath 1999). However Vollrath (1999) states there are limits to a generalist and the fact that many spiders have evolved seven types of silk glands indicates that there is the necessary genetic diversity to produce a new, radically different silk, if the need arises.

1.2.2. Silk

Spider silk is used for a variety of different purposes such as web spinning, cocoon construction and for depositing sperm. Spider silk is a very strong, very light material that also has remarkable ductile strength. Some types can stretch to 140% of their own length without breaking (Vollrath and Knight 2001). This is equal to commercial polyaramid (aromatic nylon) filaments which are regarded as a benchmark in synthetic materials (Vollrath and Knight 2001). Spider silk is initially in a liquid form inside the spider, and becomes solidified once exposed to the air. The weight of spider silk's liquid form is ten times less that its solid form, and whilst it is initially soluble in its liquid form, once solidified it becomes insoluble (Foelix 1996). Although not soluble when exposed to the air, silk does combine with water leading to an altering of its properties. For instance, whereas a silk thread that is dry has a stretching capacity of 30%, the equivalent wet silk thread has a

stretching capacity of 300% (Foelix 1996). These properties are likely related to the mechanics of energy absorption from prey impact into webs, as it is often the dry threads that form the basic framework of the web and therefore their elasticity should be less than those of the capture spirals themselves (Foelix 1996). Most studies on the properties of silk so far have focussed on orb-weaving spiders (Eberhard 1990). The orb weaving spiders are in the family Araneidae (marked in red in the phylogeny table).

Silks are largely composed of non-essential amino acids but the precise composition varies (Vollrath and Knight 2001). Here is a table showing an analysis of the composition of two different types of silk that illustrates these differences with data expressed as residues per 1000 (also see figure 2).

Amino Acid	Spider frame silk	Viscid silk
Glycine	372	442
Alanine	176	83
Serine	74	31
Proline	158	205
Acidic	58	30
Basic	11	31
Aromatic	45	37

The total number of 'small' amino acids (serine, alanine and glycine) are taken to be an indication of crystal forming potential (Wainwright, Biggs et al. 1982). However, different types of silk and even the same silk can show different amino acid make up (Work and Young 1987; Craig 1997), and further more it is believed that the large differences in silk's mechanical properties are the result of the way in which it is spun (Vollrath and Knight 2001) rather than simply a product of the amino acid composition.

The process of extrusion and solidification of liquid silk into the solid form has thus far not been possible to replicate *in vitro* despite the fact that it is manufactured

naturally at ambient temperatures and with water as the solvent (Vollrath and Knight 2001). Natural production of silk is a complex process involving many different organs of the spider. The apparatus that the spider uses to spin dragline silk is the major ampullate gland, which contains a long tail and a wider sac area (Vollrath and Knight 2001). It is the tail area that does most of the secretion, and the sac area that is the main storage repository (Vollrath and Knight 2001). The sac leads to a funnel and a tapering duct, which takes the form of three loops inside a sheath, is where the fibre is formed (Vollrath and Knight 2001). The fibre then undergoes further processing in the narrow tubular region, which is specialised for rapid water recovery (Vollrath and Knight 2001). The silk then exits at the spigots. However the method and speed at which the silk is moved along the process is also important in determining its composition. In the ducts, which are long, thin axially oriented structures (Vollrath and Knight 2001), convergent geometry forces the liquid silk (dope) through at a constant slow rate, which ensures low and uniform stress on the fibres (Vollrath and Knight 2001). Then there is high stress in the internal drawdown tuber, where the thread pulls away from the walls of the third limp and this process is believed to bring dope molecules into alignment. Silk proteins then crystallise and aggregate, while taking on a hydrophobic property (Vollrath and Knight 2001). The increased hydrophobia leads to phase separation and loss of water from the thread, leading to solidification. To assist phase separation, hydrogen ions are secreted in the third limp of the duct, also rendering the dope section more acidic (Vollrath and Knight 2001).

Studies on a range of different silks have established that the general consensus structure is a sequence of amino acids that undergo self-assemblage to a beta sheet conformation. These beta sheet blocks are separated by amino acid segments with bulky side groups. The stack of beta sheets form crystals, and the other segments form amorphous domains. It has been proposed that interactions between the strained elastic semi amorphous regions and the strong crystalline

segments gives silk its elastic and tensile properties (Liu, Sponner et al. 2008; Ene, Papadopoulos et al. 2009). Our understanding of silk structure is not fully complete however, and the beta-sheet crystal formation has been challenged by observations of random coil B turns, parallel and anti-parallel B-pleated sheet crystals, A-helices or the more compact 3 (1) helices (Gosline, Denny et al. 1984; Gosline, Pollak et al. 1994; Termonia 1994; Kummerlen, vanBeek et al. 1996; Simmons, Michal et al. 1996; Case and Thornton 1999).

1.2.3. Types of spider silk

Spider silk falls into 5 basic categories: major ampullate, capture spiral, tubiliform, aciniform and minor-ampullate. Dragline silk is used for the web's outer rim and spokes, and also for the lifeline. The lifeline is a strand of silk that the spider trails (or drags) behind it, the purpose of it being that in the event that the spider should get knocked or blown off an edge, the spider can climb back up the lifeline to relative safety. Being used this way requires that dragline silk is stretchy and strong. Dragline silks are described as being unmatched in their tensile strength (200,000psi) and elasticity of 35% (Denny 1976).

Capture spiral silk is used for the capture lines on the web. It is sticky, extremely stretchy and tough. As its name suggests, it is used for capturing prey as they crash into the web; and because of this it needs the qualities of stickiness, stretchiness, and toughness to prevent breakage when absorbing the impact energies of prey. The tensile strength of silk has been estimated at 1 Gpa (Opell and Bond 2000) and its stretching limit has been estimated at between 500% and 1000% (Vollrath and Edmonds 1989; Kohler and Vollrath 1995).

Tubiliform silk is the stiffest type of silk and is used for protective egg sacks. As it is used for egg sacs tubiliform silk requires different properties to other silks and there is less advantage in it being highly elastic. But conversely there is a greater advantage for the spider if this silk is able to resist decomposition. Unlike the other types of silk, tubiliform silk is not secreted daily, but must be sufficiently robust to resist predators, parasites and fluctuations in temperature (Zhao, Zhao et al. 2005)

Aciniform silk is used to wrap and secure captured prey, and is two or three times stronger than the other types of silk. It is not fully understood why wrapping silk should require such strength, although it been noted that wrapped prey decomposes less rapidly than unwrapped prey which suggests that it has some method of resisting and prevent decomposition by microbes (Zortea and Fischer 2009). Aciniform silk has been calculated to be 50% tougher than major ampullate silk, have four times greater expendability and be ten times thinner, yet it does have less ultimate strength (Hayashi, Blackledge et al. 2004).

Minor-ampullate silk is used for temporary scaffolding during the construction of webs. Unlike other silks, minor-ampullate silk irreversibly deforms when stretched (Liivak, Flores et al. 1997), likely because it is not required as a permanent structure. At the molecular level it also differs from other silks in that the proteins that make it up are non repeating in terms of their amino acid composition (Liivak, Flores et al. 1997).

Of the five types of silk, tubiliform and aciniform silks would seem to be the most likely to be antimicrobial. Tubiliform silk is used to protect the eggs of the spider and would benefit the spider if it was able to resist microbes. Aciniform silk is used in prey wrapping and this silk being antimicrobial would benefit the spider as the prey would be less likely to contain microbes. More of the wrapped prey would remain if not subject to microbes. Capture spiral silk being able to resist

decomposition by microbes would benefit the spider both by reducing the amount of resources the spider devotes to web manufacture and also by reducing the amount of microbes the spider comes into contact with on the web. Antimicrobial properties of dragline and minor-ampullate silk, as they are often used temporarily, appears to offer fewer benefits to the spider.

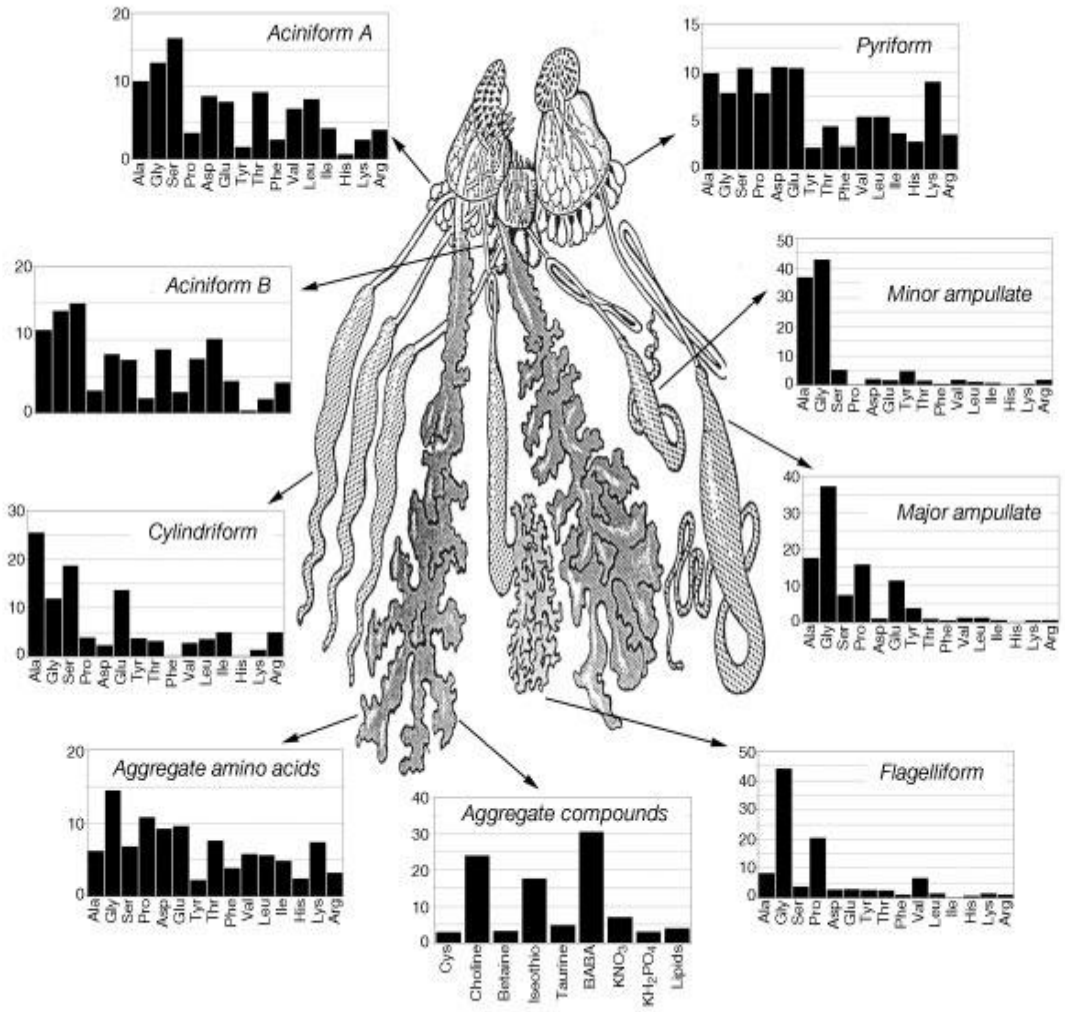


Figure 2 Diagram showing the proportions of amino acids in different types of silk, taken from (Vollrath and Knight 2001)

1.2.4. Spider uses of spider silk

Spiders use silk for a great variety of purposes, and probably the most well known, and certainly noticeable, is web manufacture. The most commonly recognised webs are those of the orb weavers, which produce "circular" spiral webs which tend to be aligned on a vertical plane, although some examples of horizontal orb webs do exist for example from the spiders *Octonoba sybotides, Araneus marmoreus, Leucauge venusta* (Opell 1998). A notable aspect of orb web spiders is that they tend to construct and then redigest their webs every single day. Other longer lasting webs include the sheet web, such as that produced by linyphiid spiders (Benjamin and Zschokke 2004) which is rather less elegant than the orb webs and consists of a three dimensional "mesh" of various interlocking threads. Another type of web also used to assist in prey capture is the funnel webs, where the spider constructs a thick layer of silk which forms a funnel shape and a flat base which spreads out from the funnel. The spider hides in the narrow funnel part, waiting for prey to land upon the flat base area when it pounces out and subdues the prey. This type of web is spun by spiders such as *Tegenaria domestica, Agelena limbata* and *Hololena curta* (Roland 1983; Masumoto 1993; McCormick, Li et al. 1999). Apart from for the purpose of prey capture, many spiders also make their webs as a retreat to protect them from the external environment and predators. Anecdotally it has been observed that the majority of cobwebs (spider webs that are no longer occupied) tend to be sheet or funnel webs rather than orb webs, although this could be due to their more robust structure.

Other uses of silk include egg sac construction by females which involves the spider manufacturing a ball of silk with the egg in its centre for protection. Sex specificity of particular types of silk is also found in males, which produce silk for "sperm webs" in which a bundle of sperm is wrapped in protective silk before being inserted into the female.

Prey wrapping is another use of silk. The spider subdues its prey and then wraps it up in a parcel of silk, wrapping the prey many times over. It has previously been observed that wrapped prey decomposes less rapidly than unwrapped prey (Zortea and Fischer 2009).

There are also more extreme uses of silk that have evolved in particular species. For instance, Bolas spiders use a silken lasso to hunt passing moths and, net casting spiders make a net in which prey passing underneath are caught and *Argyroneta* "diving bell" spiders produce a silken "diving bell" underwater by making a ball of silk and trapping air inside.

Another interesting, if fairly common use of silk amongst the spiders is that of ballooning, whereby the spiders cast a trail of silk before taking off in the wind using the silk as a "parachute" to disperse them far. It has also recently been observed that certain species of spiders use silk as an "anchor" against the wind when the spider has landed in water (Hayashi 2010).

1.2.5. Human uses of spider silk

There are records indicating that spider silk has historically been used by humans and it is now the subject of research into biotechnological applications. One traditional use, and one closely related to this thesis is that peasants in the Carpathian Mountains applied sections of the tubular shaped webs of *Atypus* spiders as topical bandages to heal wounds. This was believed to be beneficial due to the antiseptic properties of the spider silk (Heimer 1988). Other traditional uses include *Nephila* webs being used to catch small fish (Heimer 1988). In certain areas in Madagascar, spider silk has also been observed being used in bag and clothing

construction. Spider silk's very regular, narrow structure has also allowed it to be adapted for use a crosshair in microscopes and telescopes (Berenbaum 1995).

Modern uses of spider silk have involved using *Nephila clavipes* silk to help in mammalian neuronal regeneration (Allmeling, Jokuszies et al. 2006). This study also found that spider silk did not appear to provoke an auto immune response in human cells. This is an important point to raise because, if a material is to be used therapeutically then it is important that the material is neither toxic nor swiftly destroyed by human immune systems, negating any potential beneficial effects. Another recent biotechnological application of spider silk described is for using recombinant spider silk particles as drug delivery vehicles (Lammel, Schwab et al. 2010) This study found that a constant drug release rate could be realised for a period of two weeks using spider silk, and concluded that spider silk particles have high potential to be used for diverse applications when there is a requirement for controlled release from biodegradable carriers. Spider silk has also been suggested to be used as a load bearing biomaterial by Brown (2011) because of its biocompatibility and strength and toughness although the work indicated that further work would be required before the full potential of silk can be realised.

For future potential uses with applications ranging as wide from artificial tendons to rust free panels have been suggested (Jadhav 2009). Spider silk has been suggested as a suitable replacement material for many existing products such as clothing, ropes, seat belts, body armour, parachutes and biodegradable bottles, all of which could show both cost and environmental benefits if made from spider silk rather than current manmade materials (Jadhav 2009).

1.3.1. Habitats of spiders

Spiders live in a very wide variety of habitats, and different species reside at such diverse locations as in the freezing Arctic to the boiling and dry equatorial desert regions (Foelix 1996). While spiders are probably most abundant in areas of rich vegetation, certain species favour habitats far removed from this, such as tidal zones and sand dunes (Lamoral 1968). The differing habitats that spiders reside in means different types of silk are required for optimal survival of the spider.

Spiders also show large variety of habitat variation in that there are certain genera of spiders that live in water habitats, such as *Dolomedes*, but generally spiders are most comfortable in land environments, and it has even been suggested by Turnbull (1973) that all niches on land may have been conquered by spiders.

In rich vegetation areas, which is the habitat most suitable for spiders, it has been suggested Duffey (1966) that the environment can be split into four basic strata; soil, field, bush and tree levels, and correspondingly spiders that live in these habitats have been classified in similar strata (Toft 1978). For example, the wolf spider *Paradosa pullata* lives very close to the ground, normally residing at a height of 0-5cm (Duffey 1966). Also, many sheet web spiders of the family *Linyphiidae* reside near the ground, which is of interest to this project as it is possible that spider webs that are on or close to the ground would be likely to posses more antimicrobial compounds, or would at least have more to potentially gain from having more antimicrobial compounds because of the high occurrence of microbes on the ground (Stotzky 1997). Conversely it could be expected that orb web spiders, which build their webs off the ground, would be less likely to possess antimicrobial properties due to their webs coming into less contact with microbes.

1.3.2. Life cycle of spiders

As with morphology and behaviour, spiders show a variety of life cycles, but there are some features common to nearly all, if not all species of spider. The vast majority of spiders start off as fertilised eggs, which are protected by a silken sac (Roberts 1995). The egg is protected by the silken sac, which is made of tubiliform silk.

While still in the egg the spider grows, usually obtaining nourishment from the yolk, into a pre-larval stage. This stage is largely immobile and the spider must moult to enter the larval stage (Roberts 1995). The spider then moults to a nymph stage, and now will have the basic body plan of a spider. In many species the nymph will still be in the egg case but it is often at this stage that the spider will first hunt prey, in many cases this will be other spiderlings (Roberts 1995). The spiderling will then leave the egg case and enter the outside world. In many species there is no further parental care, but conversely many species still show high levels parental investment, *Theridiiae* spiders for instance, regurgitate food for their young (Roberts 1995). Bilde and Lubin (2001) documented kin caniballism in the social spider *Stegodyphus lineatus* and Salomon, Schneider et al. (2005) recorded suicidal maternal investment in this species.

The next stage tends to be dispersal, with some spiders simply going to the closest viable spot that is nearby, and other species travelling much further by a process called “ballooning”, where the spider creates a “kite” out of silk and takes off in the wind, sometimes travelling thousands of miles by this process, although it seems a few kilometres is a more common ballooning distance (Roberts 1995). The kite is made out of dragline silk, and once the spider has landed is detached from the spider.

After dispersal from their birth place spiders undergo the process of hunting, growing and moulting. The spider's silk is commonly used in hunting, to aid the capture of prey. Moulting is highly dangerous for the spider both due to predation and to simply being unable to free themselves from the moult, so many species try to find secure locations for moulting (Roberts 1995). Often the spider will retreat into its web to moult, which has been constructed to be a secure retreat by the spider.

Once the spider has reached adulthood, it starts to undertake reproduction, normally this starts with the male seeking out the female, often detected by pheromones (Roberts 1995). Once the male has located a female, courtship follows and the male will use many different senses to insure he is not mistaken as prey (Roberts 1995). Male spiders will collect sperm on their palps and then the sperm is protected in a sperm web made of silk before insertion in the female.

The longevity of spiders varies, although most temperate species live for one year (Foelix 1996). However certain *Atypus* species will live for seven years and some Mygalomorphae females can live for up to twenty years (Foelix 1996).

1.3.3. References to spider silk possessing antimicrobial properties.

This study investigates one particular property of spider silk, namely its potential to act as an antimicrobial agent. There are currently very few peer reviewed, published trials testing for antimicrobial properties of spider silk. Heimer (1988), in a general book on spiders, mentions that microbes are unable to grow on spider silk and attributes this to the silk having a acidic property, but this appears to be the result of speculation and not findings from an experiment. Borders (2001) gives details of a school project that investigated spider silk's antimicrobial properties, but these data were inconclusive. Chakraborty (2009) submitted an abstract to the

European Congress of Clinical Microbiology and Infectious Diseases in 2009, saying the dissolved proteins of spider silk possessed antimicrobial properties, but it appears that these findings were not published further.

1.3.4. *Atypus* silk as a bandage

Atypus silk has been recorded as being used as a bandage for wounds by peasants living in the Carpathia mountains (Heimer 1988). The silk reportedly facilitated healing and even joined with the skin. A good material for a bandage might have good water absorbance as some bandages soak up the dampness around the wound, making the conditions less ideal for microbe growth. Conversely water proofing is also important as it prevents materials from the outside entering the wound.

1.4.1. History of Antimicrobials

E.coli is a gram negative bacteria that is part of the Enterobacteriaceae family of gamma-proteobacteria (Ravcheev, Gerasimova et al. 2007) and was discovered by Theodor Escherich in 1885. *E. coli* resides in the lower intestine of many warm blooded organisms (Bentley and Meganathan 1982) and is often a benefit to the host by producing vitamin k_2. *E. coli*'s ability to survive outside its usual host organism, as well as the ease in which it can be manipulated genetically, makes it an excellent research organism (Lee 1996).

B. subtilis is a gram positive bacteria commonly found in soil (Madigan M 2005). It is a member of the Bacillaceae family and was described by Ehrenberg in 1835. Like *E. coli*, *B. subtilis* is a heavily studied organism partly because of the ease of genetic manipulation (Youngman, Poth et al. 1989) it is often used as the Gram-positive equivalent in studies of the Gram negative *E. coli* (Sheu, Salomon et al. 1975).

Saccharomyces cerevisiae is a yeast fungus of the family Saccharomycetaceae. Since ancient times, where it was believed to have been originally harvested from grape skins (Vaughan-Martini and Martini 1995) *S. cerevisiae* has been used extensively by humans, in brewing and baking processes and now is very commonly used in biological research (Hoekstra 1998).

Antimicrobials have been used by man since pre-history times, but it was only after Pasteur and Joubert's observations that the massive potential applications started to become realised. Pasteur stated "if we could intervene in the antagonism observed between some bacteria, it would offer 'perhaps the greatest hopes for therapeutics" (Kingston 2008) and the search for antimicrobials that could benefit humans began in earnest. The first recorded antimicrobial compound was

discovered by Ehrlich in 1909 and called 606 compound, so called because he discovered it after his 606th animal experiment (Jones and Ricke 2003). With this discovery previously fatal diseases, like syphilis, became treatable. However the reaction was not quite as positive from the medical community as one might expect, with many physicians happy to continue to treat and receive payment from the patients until death (Jones and Ricke 2003). Another breakthrough came in 1932 when Domagck, who had some success treating mice with fungal infections with *protosil rubrum*, but had yet to test it on humans; however when his six year old daughter became ill, he decided to test it on her, and she quickly recovered. After repeat experiments on other humans the compound was decided to be safe and effective was treatment of fungal diseases. With the discovery of penicillin and tetracycline in the 1940s previously illness like gonorrhoea *Streptococcus* throat infections, or pneumonia, which could lead to very serious complications, became easily treatable with short courses of antimicrobials.

1.4.2. Search for Antimicrobials

While many different types of antibiotics were discovered quite rapidly after the initial finds in the mid 20th century, progress has slowed and no new antibiotics effective against gram negative bacteria have been discovered this decade (Norrby, Nord et al. 2005). There is doubt about the future effectiveness of antimicrobials because microbes are developing resistance to the antimicrobials currently used. Multidrug resistance is of particular concern (Cohen 1992; Neu 1992). While there are some fungal strains with multi-resistance, the problem appears less widespread, possibly because fungi do not horizontally transfer genes for drug resistance between species in the same way as bacteria do (Norrby, Nord et al. 2005).

Previously antimicrobials were screened for from natural sources such as soil and for the years 1983-1994 78% of new drugs came from natural sources (Cragg,

Newman et al. 1997), but now new antibiotics are searched for by examining the mechanics of microbes to identify cellular processes that do not occur in humans as targets for new antimicrobial drugs (Norrby, Nord et al. 2005). It has been estimated that only 20% of potential target actions have as yet been exploited by antimicrobials (Kleinberg and Wanke 1995).

There are several different methods used to test the antimicrobial properties of both natural and manmade materials. A method known as AATCC 100-1999 (Chun, Foulk et al. 2009) tests the antimicrobial properties of a material by leaving the tested material to soak in bacteria for a period of time. The material is subsequently removed and placed in a liquid growth medium. The underlying principle is that if the material is antimicrobial, then bacteria that are stuck to the material, will have been killed or at least their growth inhibited and this will become apparent when they are transferred to the growth medium.

A method that uses agar plates involves creating a plate of agar, then spreading some bacteria over the plate before placing the tested material on the plate. If the tested material is antimicrobial then there will be a zone of inhibition around the material. However Benkendorff (1999) documents weaknesses in this method, for incidence Spooner and Sykes (1972) state that many of the compounds are only antimicrobial when undissolved, but the agar plate method requires the compounds to be soluble and not highly hydrophobic.

The method used for this project involved having some liquid broth, then adding the tested material, then adding a small microbial sample. The mix is left time to grow (typically around 24 hours) and then the growth of the microbes is observed by seeing how much light absorbance a sample of the mix has. The theory behind this is that if there is an inhibitory affect of the material, then the microbial sample mixed with it will show less growth.

1.4.3. Antimicrobials from Protostomes

To increase their chances of survival, all organisms have some form of defence against attack from foreign bodies. A common form of attack from foreign bodies come from microbes, where single-celled organisms reside in the host's body, where if left to remain can cause disease and ultimately death in the host. Across all life organisms identify and subdue these invaders however many different processes and compounds are used for defence. The vertebrates' immune system could be deemed the most advanced immune system amongst animals (Beck and Habicht 1996). Vertebrates have many defence systems but perhaps the most sophisticated are the immune defences provided lymphocytes and antibodies, these are compounds that are tailored specifically to the invading organism. While because of this specificity there is a slight initial delay in their production, lymphocytes and antibodies have the crucial advantage to there being a "memory" of the invading species, in that subsequent invasions by the same species will be dealt with much more quickly (Beck and Habicht 1996). Invertebrates lack antibodies and lymphocytes and it has been showed that they lack a "memory" of invading species. This is illustrated clearly by experiments such as those with starfish, which show that the rejection of foreign bodies does not decrease in time with repeated invasion of the foreign bodies – in contrast to vertebrates which show a decrease in time with repeated invasions (Beck and Habicht 1996). While lacking the advanced immune systems of vertebrates, invertebrates still possess many methods of defending themselves against microbes. Lacking antibodies that are able to deal with specific threats as they come, invertebrates have to rely more on producing more general antimicrobial compounds.

Possibly because invertebrates do not have the advanced immune system of vertebrates, it was initially thought that they do not have an immune system at all.

However in 1882 a Russian scientist called Metchnikoff inserted a rose thorn in a starfish larva, and upon observing it the next day noticed that a clump of cells had group round the thorn in a process called phagocytosis, previously thought to be unique to humans, that protect the rest of organism from the foreign body (Beck and Habicht 1996). It was then seen that phagocytosis is possibly one of the most ancient immune response of organisms (Beck and Habicht 1996). This observation has led to a greater examination of immune systems and it was found that many processes thought unique to vertebrates had very similar counterparts in the invertebrates.

Common across the invertebrates are lectins, which act by binding to sugar molecules and the foreign bodies, forcing them to clump together. This acts as a kind of "tagging" system to aid the identification of the foreign bodies (Beck and Habicht 1996). Once identified, the foreign objects are encapsulated by prophenoloxidase, preventing further harm, in a process similar to vertebrates complement system (Beck and Habicht 1996).

Most relevant to this project is the use of antimicrobial peptides by compounds produced by invertebrates to inhibit the growth of microbes. Surprisingly little attention has been paid to this area (Beck and Habicht 1996) despite the potential therapeutic benefits. The first finding of an antimicrobial peptide was in the silk moth *Hyalaphora cenropia* (Boman, Nilssonf.I et al. 1974) and while the antimicrobial peptides of invertebrates tend to be released from the blood (Beck and Habicht 1996), there is a growing number of examples where the peptides are from other, sometimes externally deposited surfaces.

While there are many examples of antimicrobials derived from invertebrates (Bulet and Stocklin 2005), these tend to come from within the invertebrate's body or egg cases e.g. (Vander Meer and Morel 1995; Lehrer and Ganz 1999; Pankewitz,

Zöllmer et al. 2007), and not from external structures. Commonly, research on spider antimicrobials has focussed on spider venom (Dubovskii, Volynsky et al. 2006; Kozlov, Vassilevski et al. 2006; Pukala, Boland et al. 2007). Although snail Mucin is well documented to have antimicrobial properties (Tincu and Taylor 2004), there is a lack of study of other invertebrate residues having antimicrobial properties.

Benkendorff, Beardmore et al. (1999) and Benkendorff (1999) examined molluscs and detected evidence that both the slime and egg masses processed antimicrobial properties. While they found these antimicrobial peptides effective against bacteria from a wide variety of snails, some snail species egg masses were ineffective against fungi (Benkendorff 1999). It was also found, in relation to time, that the antimicrobial property of the egg masses decreased as the embryo developed (Benkendorff 1999). Raspotnig, Fauler et al. (2005) investigated the antimicrobial compounds present in the scent gland secretions of harvestmen *Cyphopkhal opilionid.* They found that the two species investigated were nearly visually indistinguishable, but showed a large difference in the chemical composition of the scent gland compounds. Using the disk diffusion method, they found evidence to suggest that the scent glands had antimicrobial properties.

1.5.1. Aims

The aim of this project was to investigate if spider silk possessed antimicrobial properties. The hypothesis tested was that by adding spider silk to colonies of microbes, there would be less growth of the microbes when compared with the control. There are a few studies (Heimer 1988; Borders 2001; Chakraborty D. 2009) that have hinted at this property of spider silk but the evidence is inconclusive. Additionally the project also aimed to investigate the nature of the antimicrobial compounds present on the spider silk. The project also aimed to investigate the

therapeutic potential of antimicrobial compounds in spider silk by using them to challenge mammalian cell cultures.

Based on the usage of *Atypus* silk as a bandage in the Carpathia Mountains (Heimer 1988) the project also aimed to investigate whether *Atypus* silk possess properties than would make it beneficial to be used as a wound dressing.

Methods

2.1.1. Introduction and general methods

Collection and storage of spiders

The spiders used in the project were gathered from a variety of locations

Several spiders from the genera *Tegenaria, Zilla, Araneus* and Linyphiidae used for the research were obtained from the grounds of Nottingham University, University Park Campus. Spiders were collected in one of three ways:

1. A sample of leaf litter was collected from the ground and returned to the lab. Once in the lab the leaf litter was carefully combed through to find any suitable spiders.
2. A large, sturdy plastic bag was placed tightly around several braches of a tree and then the tree was shaken vigorously. This lead to many small invertebrates coming loose from the tree and falling into the bag. Once loose in the bag the spiders were easy to spot and capture.
3. For the larger web-weavers, some were observed while around the campus and caught on their webs with containers.

Obtaining spiders from varied habitats and with a range of techniques meant a range of different species was collected.

Other sample spiders were caught in household locations. These were captured by hand before being transported to the laboratory.

Two other types of spider were also included. First, captively bred tarantulas (*Theraphosids,* from the Mygalomorphae) and a species of Scandinavian Linyphiidae, *Pityohyphantes phrygianus.*

While it was necessary to act quickly to capture the spiders and prevent them getting to their retreat – often in small nooks and crannies – care was taken to ensure the spiders were not damaged or made infirm during capture. The main danger was one or more of the spider's legs getting trapped outside the container and getting torn off or damaged. This was alleviated by ensuring the container was placed gently over the spider. If any legs were outside the container would be slightly tilted and the spider would instinctively draw its legs in.

Once captured, the spiders were placed in either a sterile 20ml universal tube, or a sterile magenta box, depending on the size of the spider. As the spiders were often reluctant, or unable, to build webs in the universals or magenta boxes when they were empty, sterile sticks 2 to 3 inches in length were added to which the spider would anchor the web around. In the universal tubes two sticks were sellotaped together and were placed leaning against the sides of the tube. For the spiders in magenta boxes two sticks were sellotaped end to end, effectively creating one long stick, and two of these longer sticks were then laid across each other in an X shape, against one of the side walls. How the sticks were used would vary with the type of web the spider tended to weave. For the orb-weavers the high point of the sticks would generally be used as a point from where the spider would start its radial threads. For the funnel web spiders, the bottom of the sticks was used as the bottom of the funnel web, which was also connected to the corners of the magenta boxes. The sheet web spiders and the Mygalomorphs, seemed to use the sticks as a "starting" point for the web. Overall it appeared that the spiders were more

conformable attaching their webs to the relatively abrasive sticks, compared with the smooth plastic sides of the universal tubes and magenta boxes.

As the project involved working with bacteria, it was important that there were as few contaminates as possible as to not interfere with the bacteria being examined. This is why sterile sticks and containers were used rather than storing the spiders in conditions that would be closer to their natural environment, for example, filling the pots with soil, as the bacteria present on the soil might contaminate the experiment.

To maintain air circulation while still preventing escape, the lids of the universal tubes were removed and the top of the tubes were carefully sealed, with cotton wool plugs. For the spiders in the magenta boxes, a small hole was pieced in the roof to enable air flow. Ensuring the air was well circulated meant the spiders had fresh air to breathe and also prevented the formation of mould by reducing humidity.

The spiders were kept hydrated by spraying the spider and its container with a fine mist of water every 3-4 days. A standard watering can was used for this purpose, set on the finest spray setting. A fine spray setting was used because there was a danger of the spiders downing in larger droplets due to being unable to escape the surface tension of the water.

For feeding, the spiders were fed *Drosophila* roughly every 1-2 weeks with the larger spiders being fed greater individual numbers of flies and occasionally with crickets. Flies would be placed in a -20°C freezer for the 5 minutes before feeding in order to sedate them so they could be easily tipped into the spider containers. The crickets would be individually captured using fine forceps and dropped into the spider containers.

To identify the species captured, the established key produced by (Roberts 1995) was used as a starting point, sometimes with more specialised sources of literature used to get the precise species.

2.1.2. Microbe collection and storage

The two species of bacteria, *E. coli* and *B. subtilis*, were obtained from the microbiology department of Nottingham University. The bacteria were stored in liquid broth (ingredients listed below) at 8°C in 20ml universals. The two species of fungi, *A. niger* and *S. cerevisiae*, were obtained from the fungal biology department of Nottingham University. The fungi were stored on solid agar growth medium at room temperature (ingredients listed below). Jurkat mammalian cells that were used were obtained from and maintained in the mammal cell culture labs at Nottingham University in mammal cell growth medium (ingredients listed below).

2.1.3. Obtaining the silk from the spiders

Web silk was taken from spider containers in which individuals would naturally spin silk without any external input needed, although the sterile sticks were often used by the spiders to anchor the silk. To collect the silk sterile pipettes were used to gather the silk, then the pipettes, with silk attached, were placed in the growth medium. To collect the silk, the pipettes would simply be run through the middle of the silk threads, where the silk would stick to the pipette, additionally the silk was sometimes wound round the pipette to enable a larger sample to be gathered. While attempts were made to obtain approximately the same amount of silk per trial, the large variation in the silk density of the webs made this difficult. Efforts were made to try and disturb the spider as little as possible, although some disruption was unavoidable. Egg silk was taken from the egg case with the embryo

removed. Dragline silk was gathered by letting the spider run off an edge and then using two pipettes to "wind" up the silk while the spider still sailed down. Depending on the type of web spun, the frequency at which silk could be harvested varied. Generally the orb-weavers of the genera *Araneus* and *Zilla* would produce a web every day. Sheet web and funnel web spiders *Tegenaria* and Linyphiidae tended to produce enough silk to be harvested every two days.

2.1.4. Liquid broth method

Testing antimicrobial activity by observing growth in liquid broth

To test antimicrobial activity via optical means, a method by (Patel, Patel et al. 2009) was used.

1. 10ml of liquid broth (ingredients listed below) was poured in a sterile 20ml universal tube.

2. 50µl of the sample microbe was pipetted into the tube.

3. Silk and the sterile pipette with which it was collected or sterile pipette alone (control) were submerged in the mix of the liquid broth and microbe.

4. The tubes were placed on a shaker rotating at 150 rpm and heated to 27°C

5. At observed times a 2ml sample was pipetted from the tube and placed in a cuvette.

6. The cuvette was then placed in the Photospectrometer and the absorbance reading at 660nm (for bacteria) and 550nm (for fungi) was recorded for

both the control and spider silk tubes. Light absorbance is expected to be proportional to bacterial density in the medium with absorbance increasing as microbial density increases.

Preliminary experiments indicated that 10mls of liquid broth inoculated with 50ul of the sample microbe gave suitable growth rates over a 48 hour period.

While it would have been desirable to have the silk floating freely in the experimental broth, this proved difficult as the silk stuck to what was used to gather it. Vortexing the forceps or spatula vigorously in the broth, which could have increased the ease of detaching the silk, might have negatively affected the physical or chemical properties of the silk and was therefore not done.

An incubation temperature of 27°C was used because it lies within the natural range that spider silk would encounter in nature (varies with weather and night/day, normally between 5°C and 15°C but can reach the high 20s), and the temperature at which the microbes are typically cultured (37°C for *E. coli* and 30°C for *B. subtilis*). Experiments done in this thesis on growth curves of the bacteria at 27°C show there are satisfactory growth curves at 27°C for both *B. subtilis* and *E. coli.*

2.2.1. Agar plates

Testing antimicrobial activity by observing growth inhibition on agar plates

1. Agar (ingredients listed above) was heated in a microwave for roughly 3 minutes (exact time would depend on amount of agar) until liquid.

2. The melted agar was then poured in a standard Petri dish; the agar was poured to cover the Petri dish approximately 0.4 cm in depth

3. The agar was left to cool down for around 40 minutes until it returned to a solid.

Then, to get the bacteria across the lawn, two different methods were used:

I. 20 µl of the tested microbe was pipetted on to the centre of the plate and then spread around using the glass spreader.
II. 20 ml of the still liquid agar, once slightly cooled down to around 50°C, was mixed with a 1ml sample of the bacteria, before being poured on the agar and left to solidify. The advantage of this method over the first approach is that it gave a more consistent lawn, the disadvantage is that it often required longer for the growth to be observable.

After the agar plates were prepared with the microbe spread.

4. A stand of silk was laid down on the lawn. Depending of the size and type of silk, different methods were used.

I. For the very narrow as well as hard to see silk, the silk was collected on a sterile pipette and then both the pipette and the silk were placed on the agar plate.
II. If using egg sack silk, these were simply picked up with forceps and then placed on the agar.

III. For the web silk on the forceps it was transferred to the agar by carefully laying the silk down on the plate, although this often proved tricky as to get the silk seemed to stick stronger to the forceps than the agar.

5. Then, the plates were placed in an incubator at 37°C and left for around 12 hours if method 1 was used, and for a longer period if method 2 was used.

6. After the period to allow growth, the Petri dishes were observed. The growth of the bacteria in the areas far away from the silk was compared with the growth areas near the silk.

2.3.1. Silk treatments

To potentially identify the compounds on the spider silk that is inhibiting the growth of microbes, various treatments were used on the silk, which was then tested for its antimicrobial activity by the methods described above (2.1.3.). Digestion with Proteinase K and treatments such as heat treatment were used to establish whether the agent was behaving as if it was a protein i.e. being destroyed or denatured under the conditions applied. Soaking in water or ethanol was used to see if the agent was present on the silk surface and soluble.

1. Ultra Violet treatment: The spider silk was placed in the UV hood and subjected to UV radiation for 20 minutes.
2. Freeze treatment: The spider silk was placed in the freezer at -20c for between 1-2 hours

3. Proteinase K treatment: The silk was soaked in Proteinase K for 1-2 hours, then removed and then left to dry.
4. Proteinase K Run-off: The Proteinase K that the silk was soaked in was added to the bacterial tube.
5. Heat treatment: The spider silk was heated in a hotbox to 80°C for between 1-2 hours
6. Ethanol treatment: The silk was soaked in Ethanol for 1-2 hours, then removed and then left to dry.
7. Water treatment: The silk was soaked in Ethanol for 1-2 hours, then removed and then left to dry.
8. Soaked water: The water that the silk had been soaked in was added to the broth, with an equal amount of water added to the control tube.
9. Boiling the silk: The silk was boiled for 20 minutes in water.

2.4.1. Ampicillin resistant *E. coli*

In order to circumvent the problem of bacteria naturally present on silk "masking" the inhibition of *E. coli* or *B. subtilis* bacterial growth by the silk, an additional experimental approach was tried. Silk was treated with ampicillin to kill bacteria already present on it. This silk was then tested against ampicillin resistant *E. coli*. Two controls were used in this method. The first control had ampicillin resistant *E. coli* plus ampicillin, the second control had ampicillin plus silk. Comparison of the silk plus ampicillin plus ampicillin resistant *E. coli* sample with control one tested if the silk was having an effect. Comparison of control two with the silk alone tested if the ampicillin was having an effect on the bacteria on the silk.

This experiment was done to kill off the bacteria present on the silk, but then to test the silk against a microbe that is resistant to the treatment on the silk.

However it is worth noting that because some antibiotics have similar modes of action it is possible for a bacteria to develop resistant to one type of antibiotic, then also have resistance to an antibiotic that has a similar mode of action (Cirz rt 2006).

Bacteria have four main methods is which they are able to resist previously effective antimicrobials. One method, somewhat simpler than the other methods is that the bacteria becomes more able to tolerate the antimicrobials by both reducing its permeability and increasing its efflux of the antimicrobial compounds (Nikadio 2009). Another method by which bacteria become resistant to antimicrobials is the alteration of the antimicrobial target site, in this altered form the compound is unable to bind with the bacterial cell and so does not inhibit it (Robicsek A 2006). The resistance method used by ampicillin is the production of Beta-lactamase, which alter the antimicrobial compound, rending it either ineffective or able to bind to the bacteria (Robicsek A 2006). The fourth method is the alteration of the metabolic pathway (Neu 1982).

2.5.1. Analysis

Statistical analyses of light absorbance data were made using the sign test and the paired samples t-test. The sign test is non-parametric and does not include information on the magnitude of the difference between the two paired values. It may therefore be more conservative than the paired samples t-test, which assumes a normal distribution of values. In this case there was an *a priori* prediction that silk would reduce growth therefore test was 1 tailed.

The paired t-test assumes homogeneity of variance and while the samples with silk showed greater variance than the control samples, this was not found to be significantly different using Levene's test of equality of variance ($p=0.5$).

2.6.1.Calibration

To convert the readings of the spectrometer into bacterial counts it was necessary to calculate roughly how many bacteria per ml equalled a spectrometer reading. This was done by growing the bacteria and checking the spectrometer readings every hour, then 20ul was taken from the microbes, and diluted to various degrees, depending on the amount of bacteria likely to be present. This diluted sample was then spread on an agar plate as described above for the agar plate inhibition test. The plates were left overnight and then the number of colonies on the plate was counted.

The figures were subject to regression analysis to see if a linear relationship provides a strong correlation between the spectrophotometer reading and number of *E. coli*.

Equation	R^2	P Value
Linear	0.723	P < 0.0001

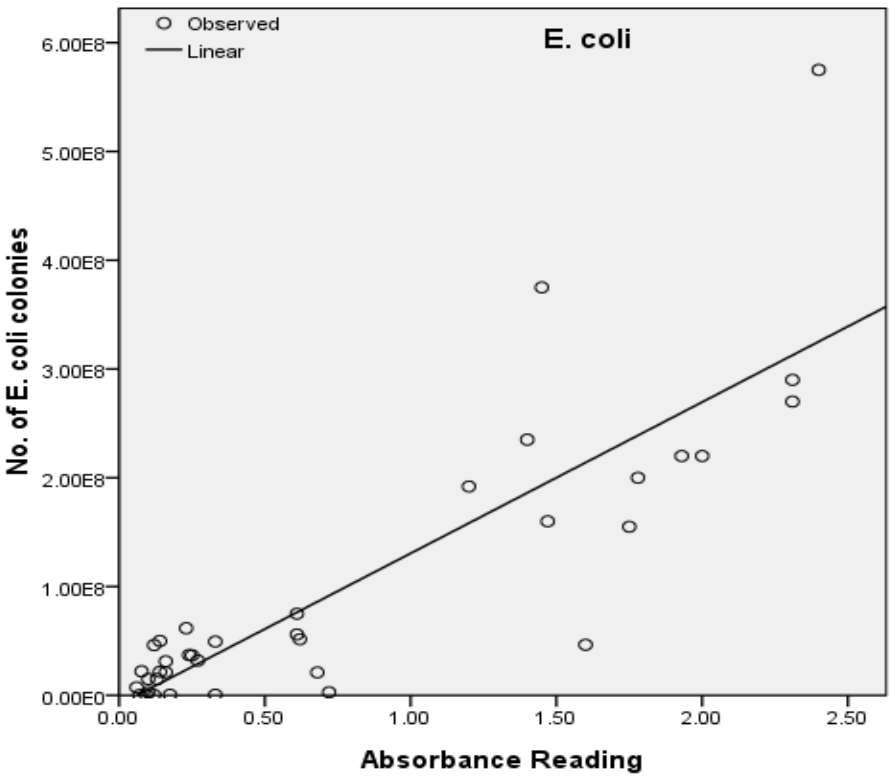


Figure 3.

The figures were subject to regression analysis to see if a linear relationship provides a strong correlation between the spectrophotometer reading and number of *B. subtilis*.

Equation	R^2	P Value
Linear	0.695	P < 0.0001

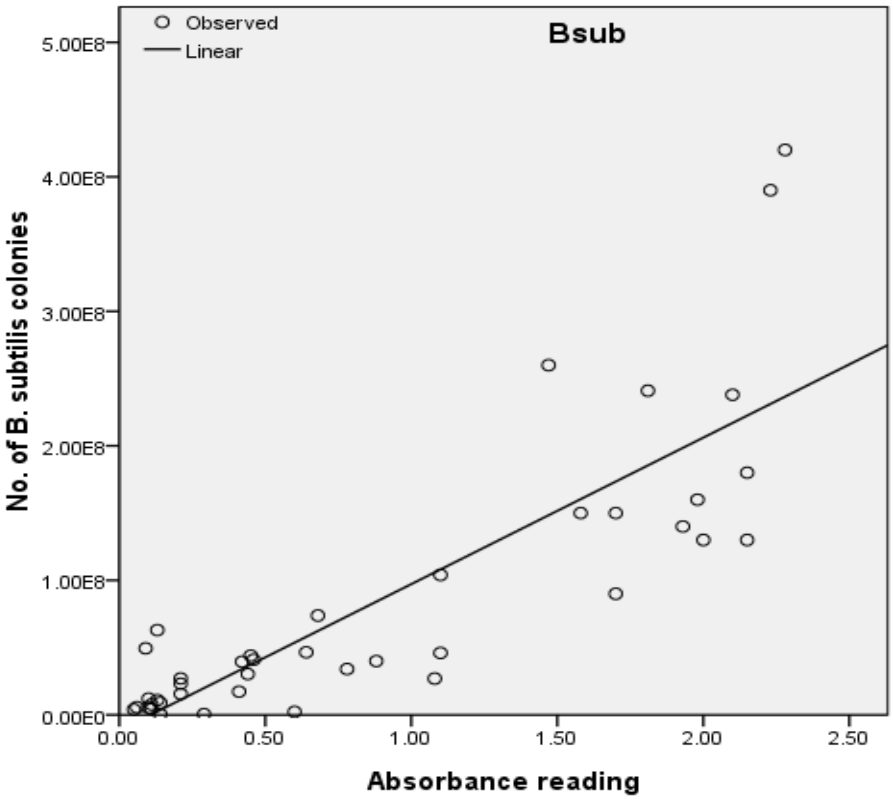


Figure 4.

Using these equations an estimate of the number of bacteria present in each sample can be produced.

2.7.1. Testing the effect of spider silk on mammal cell growth

Jurkat cells are an immortalized cell line of T lymphocyte cells that originally came from the peripheral blood of a 14 year old boy who suffered from T cell leukaemia. The Jurkat cells are used to study various mammal cell processes but especially the susceptibility of cancers to drugs and radiation.

Jurkat cells are relevant in this project with relation to the antimicrobial affects of spider silk. When an antimicrobial compound is discovered, a natural next step is to see if the compound could have potential health benefits for humans. If a compound is potentially beneficial to humans it needs to be known if the compound is both not going to interfere with mammal cells negatively, and also that the compound doesn't provoke an immune response in mammals and so is quickly destroyed. Allmeling, Jokuszies et al.(2006) tested spider silk in assisting with mammal neurological regeneration and found no evidence of an immune response attacking the spider silk. This experiment examined the other potential stumbling block and looked if spider silk would affect the growth of mammal cells.

Jurkat cells were removed from liquid nitrogen storage and quickly placed in a water bath at 37°C. After heating for around 10 minutes, the cells were added to 5ml of growth medium and placed in the incubator at 37°C and 5% CO_2. They were then left to grow for two days before being removed and the number of cells per ml calculated by removing 20µl of cell suspension, adding 20ul of a dye, Trypan Blue, and then taking 20µl of this mix and placing it between a haemocytometer and a cover slip. Using the grids on the haemocytometer the number of cells was counted.

Because the mammal cells are highly susceptible to bacterial infections, the spider silk used was subjected to 20 minutes of UV light in order to kill any bacteria that might be on the silk and ensure that the experiment measures the affect of spider silk of mammal cells, rather than spider silk plus contaminates on mammal cells.

These conditions had already been shown not to destroy the antimicrobial activity of the silk.

Two samples of 5ml of growth media was used and then approximately 3 X 10^5 cells (amount calculated via amount per ml) was added to both samples. One of the samples had silk added via the pipette method, whereas the control just had a pipette added. These were then placed in the 37°C 5% CO_2 incubator overnight.

The samples were then gathered and then the total number of cells was counted in the same way as before. The pH of the samples was also recorded.

2.8.1. *Atypus* silk as a bandage

Water absorbance:

1.5ml of water was poured into an Eppendorf tube and the total weighed, a sample of *Atypus* silk approximately 1.5cm^2 in area was weighed before the silk was placed in the tube. Then the silk was added to the eppendorf and after weighing was left for 24 hours. The whole sample (silk + water + eppendorf tube) was then weighed to see if there had been any evaporation. The silk was then removed and the tube plus water were weighed, then the silk was weighed, finally the silk was blotted with filter disks to remove any large drops of water and then weighed again.

Water proofing:

Roughly 1.5cm^2 of *Atypus* silk was sellotaped around the top of a 0.5ml eppendorf tube which had the lid removed, so that the silk alone covered the top. A control using the same size of cotton wool as the cover was prepared. Both tubes were then taped to the insides of a 25ml beaker with the top of the tubes facing upwards.

The beaker was then filled with water and left 24 hours. After 24 hours the water was removed from the beaker and the eppendorf tubes were inspected to see if the water was able to soak through the cover.

Microbe proofing:

1.5cm^2 of *Atypus* Silk was laid out flat before 0.1ml of *E. coli* growing in liquid broth was pipetted on to the top. This was left 24 hours before the silk was carefully laid onto a plate of agar (method and ingredients for agar in method section) with the topside facing upwards. This was placed in an incubator at 37°C for 12 hours before being removed. The agar was then examined to see if any *E. coli* had soaked though the silk and grown on the agar. The silk was then carefully removed and directly under the silk was checked for *E. coli* growth.

2.9.1. Equipment

Spectrophotometer: Biochrom Libra S6

Cuvettes: Fisher plastic disposable 4.5ml capacity 10mm lightpath

Universals: Sterilin Universal Containers Polystyrene 30ml

Boxes: Magenta GA7 Polycarbonate Height 97mm, length 77mm, width 77mm

Petri dishes: Sterilin 90mm single vent

2.9.2. Microbe strains

Aspergillus niger wild type N402 (Bos, Debets et al. 1988)

Saccharomyces cerevisiae BY4741 (Brachmann, Davies et al. 1998)

Escherichia coli JM101 (Sambrook, Fritsch et al. 1989)

Bacillus subtilis non-sporulating strain described by (Stevens and Errington 1990)

Ampiciliin resistant *Escherichia coli* pBlueScript II SK(+)(Stratagene)

2.9.3. Growth media

The *E. coli* and *B. subtilis* were grown in a liquid broth with the ingredients listed below.

1Litre LB (luria Bertani) Broth	
To 800mls dH_2O add -	10g Tryptone 5g Yeast Extract 10g NaCl
pH to 7 with NaOH	

The solid growth agar ingredients were:

1Litre LB (luria Bertani) Agar	
To 800mls dH_2O add -	10g Tryptone 5g Yeast Extract 10g NaCl 15g Agar
pH to 7 with NaOH	

The *S. cerevisiae* was grown in a broth with the ingredients listed below:

YEPD (Yeast Extract/Peptone/Dextrose):	
To 900mls dH_2O add -	20g Peptone 10g Yeast Extract 10g NaCl
Dispense 225mls per bottle.	Autoclave at 121°C.
	25mls of 20% glucose solution (autoclaved at 117°C) is added before use.

The *A. niger* was grown in a broth with the ingredients listed below:

***Aspergillus* Complete Medium (ACM):**	
To 900mls dH_2O add -	10g Glucose 1g Yeast Extract 2g Peptone 1g Casamino Acids 0.075g Adenine 10ml *Aspergillus* Vitamin solution 20ml *Aspergillus* Salt solution
pH to 6.5 with NaOH	Autoclave at 117°C.

The mammal cells were grown in the media listed below

Jurkat Cells growth medium	
To 500mls RPMI add -	Penicillin/streptomycin 5ml L-glutamine 5ml 10% FCS

2.9.4. Spiders

Tegenaria domestica

Tegenaria domestica was described by Clerck 1757 and is a member of the Agelenidae family. *T. domestica* is around 7-11mm body size and varies between a dark orange to beige colour (Roberts 1995). *T. domestica* builds a funnel web, usually in a corner, where the web consists of several layers of webs spread over a flat surface with the spider residing in a funnel in the corner (Roberts 1995). *T. domestica* is relatively long lived, which females normally living over two years, although there are incidences of *T. domestica* living over 7 years (Roberts 1995).

Tegenaria duellica

Tegenaria duellica was described by Simon 1875 and is a member of the Agelenidae family. *T. duellica* is a large domestic spider, with some male individuals reaching 75mm leg span (Roberts 1995). It is dark brown in colour and like *Tegenaria domestica*, weaves a funnel web.

Lasiodora parahybana

Lasiodora parahybana was described by Mello-Leitao in 1917. It belongs to the Mygalomorphae and is a member of the Theraphosidae family. *L. parahybana* are very large spiders, which some females reaching up to 25cm leg span and up to 100 grams in weight (Schultz 1998). *L. parahybana* produces a sheet web which the spider only repairs when damaged, however *L. parahybana* produces relatively small numbers of webs (Schultz 1998). *L. parahybana* like to live in moist, peaty ground where they are likely to be frequently exposed to both bacteria and fungi (Schultz 1998)

Araneus diadematus

Araneus diadematus is an orb weaving spider of the family Araneidae, described by Clerk 1757. Their size varies between 6-20mm for females and 5-13mm for males. They show a variety in colouration from pale brown to almost black (Katson 1972).

A. diadematus has a distinctive cross pattern on its abdomen and this is where the spider gets its common name – the cross spider. *A. diadematus* spins a relatively large, complex orb-web which can measure up to 40cm in diameter (Kohler and Vollrath 1995). It tends to spend most of its time in the centre of the web, where it can detect prey and quickly capture them. Before wrapping them (Zschokke 1996). *A. diadematus*' tend to rebuild their webs every day, and they consume the old web to conserve and re-use the proteins and molecules used in the webs manufacture (Peakall 1971).

Zilla diodia

Zilla diodia is an orb-weaving spider of the family Araneidae which was described by Walckenaer, 1802. Like other orb weavers *Z. diodia* builds and then consumes its web every day. *Z. diodia* is known for building an extremely fine web, with up to 50 radii threads (Zschokke and Vollrath 1995). The spider tends to build its web in forests and hedges and usually resides in the centre of the web (Jones 1983). For an orb weaver, it has a relatively small body, being between 2 – 4 mm in length (Roberts 1995).

Linyphiidae, a species of Linyphiidae was examined. Unfortunately the spider died and quickly degraded before more precise identification could be undertaken. Linyphiidae are a family of sheet web weaving spiders

Pityohyphantes phrygianus

The egg silk was from the species *Pityohyphantes phrygianus*, sheet web weaving spiders from the family Linyphiidae. *P. phrygianus* was described by C. L. Koch and is around 4mm in body size (Gunnarsson 1989). Its habitat is producing a web on the underside of spruce branches (Gunnarsson 1998). *P. phrygianus* produces and maintains its web over summer which it uses to catch prey and obtain nourishment, but in winter *P. phrygianus* acts as an active hunter (Gunnarsson 1985).

Results

3.1.1. *Tegenaria domestica* tested against bacteria

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.34	0.315	7.352941	+
2	0.34	0.28	17.64706	+
3	0.32	0.28	12.5	+
4	0.57	0.54	5.263158	+
5	0.5	0.54	-8	-
6	0.585	0.582	0.512821	+
7	0.575	0.57	0.869565	+
8	0.63	0.44	30.15873	+
9	1.22	0.57	53.27869	+
10	1.29	0.42	67.44186	+
11	0.61	0.56	8.196721	+
12	0.58	0.53	8.62069	+
13	0.6	0.5	16.66667	+
14	0.71	0.77	-8.4507	-
15	0.64	0.55	14.0625	+
16	1.03	1.06	-2.91262	-
17	0.95	0.96	-1.05263	-
18	2.1	2	4.761905	+

Table 1

The data for trials of silk with *E. coli* 24 incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.06	0.055	8.333333	+
2	0.15	0.07	53.33333	+
3	0.11	0.065	40.90909	+
4	0.13	0.08	38.46154	+
5	0.15	0.11	26.66667	+
6	0.07	0.065	7.142857	+
7	0.33	0.33	0	
8	0.43	0.4	6.976744	+
9	1.244	1.22	1.92926	+
10	0.33	0.35	-6.06061	-
11	0.33	0.33	0	
12	1.244	1.15	7.55627	+
13	1.244	1.19	4.340836	+
14	1.25	1.21	3.2	+
15	0.74	0.77	-4.05405	-
16	1.08	1.03	4.62963	+
17	0.88	1.15	-30.6818	-
18	0.76	1.26	-65.7895	-
19	0.89	1.18	-32.5843	-
20	0.94	1	-6.38298	-
21	0.58	0.57	1.724138	+
22	1.03	1.01	1.941748	+
23	2.5	2.5	0	
24	2.41	2.42	-0.41494	-
25	2.5	2.45	2	+

Table 2

The data for trials of silk with *B. subtilis* incubated in LB broth for 48 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.2	0.24	-20	-
2	0.2	0.26	-30	-
3	0.59	0.28	52.54237	+
4	0.27	0.29	-7.40741	-
5	0.84	0.75	10.71429	+
6	0.81	0.74	8.641975	+
7	0.57	0.51	10.52632	+
8	1.01	1.25	-23.7624	-

Table 3

The data for trials of silk with *E. coli* incubated in LB broth for 48 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	2.5	2.5	0	
2	0.12	0.1	16.66667	+
3	0.41	0.59	-43.9024	-
4	1.59	1.55	2.515723	+
5	1.59	1.42	10.69182	+
6	1.59	1.49	6.289308	+
7	1.45	1.42	2.068966	+
8	0.79	2.4	-203.797	-
9	1.12	1.2	-7.14286	-

Table 4

Entering the data for the affect on growth of *B. subtilis* after 24 hours (table 1) the samples with silk showed less growth 14 times whereas the control showed less

growth 4 times. When entered into the sign test gives a P value of 0.015. The average absorbance of the silk samples with *B. subtilis* after 24 hours was 0.64. The average of the control samples was 0.76. The 1 tailed t-test of silk inhibiting the growth of *B. subtilis* gives a P value of 0.028

Looking at the data for the affect on growth of *E. coli* after 24 hours (table 2) the samples with silk showed less growth 15 times whereas the control showed less growth 7 times. Which entered into the sign test gives a value of 0.07. However, when the silk showed more growth, it tended to show vastly higher growth and because of these samples the average growth on the silk samples was higher than the control. The average growth of the silk samples with *E. coli* after 24 hours was 0.87. The average of the control samples were 0.85. Even though the majority of samples had the silk showing lower growth, the higher average growth of the silk samples is reflected in the 1 tailed t-test value of 0.835.

Entering the data for the affect of *B. subtilis* after 48 hours (table 3) the samples with silk showed less growth 4 times whereas the control showed less growth 4 times. The 1 tailed t-test P value is 0.36

Looking at the data for the affect on growth of *E. coli* after 48 hours (table 4) the samples with silk showed less growth 5 times whereas the control showed less growth 3 times. Which entered into the sign test gives a value of 0.36. The 1 tailed t-test P value is 0.81

That *T. domestica* silk was show to significantly inhibit the growth of *B. subtilis* but not *E. coli* indicates that *T. domestica* silk is possibly effective against gram-positive bacteria (*B. subtilis)* but not against gram-negative bacteria (*E. coli).*

After 48 hours of growth, there is not significant evidence that silk is inhibiting the growth of bacteria. The data appears to show that silk inhibits the growth of *B. subtilis* after 24 hours, but that after 48 hours the silk does not appear to be inhibiting *B. subtilis* growth. It is possible that another limiting factor, most likely lack of resources, is now controlling the growth or that growth of microbes present on the silk itself are now masking any inhibitory effect.

	B. subtilis 24 Hours *T. domestica*	*B. subtilis* 48 Hours *T. domestica*	*E. coli* 24 Hours *T. domestica*	*E. coli* 48 Hours *T. domestica*
No. Trials silk sample lower growth	14	4	15	5
No. Trials silk sample higher growth	4	4	7	3
1 tailed Sign test	0.015	1*	0.07	0.36
1 tailed t-test	0.028	0.3565	0.8035	0.8065

Table 5

3.1.2. *Tegenaria domestica* silk tested against fungi

The data for trials of silk with *S. cerevisiae* incubated in LB broth for 48 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	2.5	2.48	0.8	+
2	0.4	0.2	50	+
3	2.03	2.08	-2.46305	-
4	1.97	2.01	-2.03046	-
5	2.09	2.09	0	

Table 6

The data for trials of silk with *A. niger* incubated in LB broth for 48 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	.42	.63	-50.00	-
2	2.29	2.24	2.18	+

Table 7

When tested, the *S. cerevisiae* samples (table 5) showed much quicker growth than the bacteria, so readings after 4 hours were taken. Unlike the other microbes tested, *A. niger* did not show an even spread of growth around the tube, and tended to grow in clumps. This made the absorbance readings less useful as they would vary greatly even within each sample (table 6). From these results, there does not appear to be evidence that spider silk is able to inhibit the growth of fungi. It is possible that as with observed with the *B. subtilis*, silk does have an initial

inhibitory effect on the growth of fungi, but that the inhibitory effect is only observed in the early growth stages.

T. domestica	*S. cerevisiae*	*A. niger*	All Fungi
Silk sample lower growth	2	1	3
Silk sample higher growth	2	1	3
1 tailed Sign test	1*	1*	1*
1 tailed t-test	0.2985	0.714	0.536

Table 8

3.1.3. Observations on the bacteria on the silk

Microbe	Control Absorbance Reading	Silk Sample Reading	Sign test
None	0.04	0.16	-
None	0.04	1.5	-
None	0.04	0.65	-
None	0.04	1.36	-
None	0.04	0.6	-
None	0.04	2.5	-

Table 9

When the *T. domestica* silk was placed without other substances, on the agar or in the liquid broth, bacterial growth was observed. This indicates that certain bacteria are able to survive on the silk, if not digest it. The amount of bacteria present on

the silk seemed to be highly variable, but as these bacteria are also growing in the broth it will be increasing the absorbance figures even without the test microbe growing, and hence maybe affecting the inhibition affect that the silk is having on the *E. coli* and *B. subtilis*. This growth of the other bacteria possibly had the effect of "masking" the inhibitory affect of the silk on the *E. coli* and *B. subtilis*, and experiments were done to try and calculate or ameliorate this factor.

T. domestica	No bacteria
Silk sample lower growth	0
Silk sample higher growth	6
1 tailed Sign test	0.016
1 tailed t-test	0.013

Table 10

To try and mitigate this issue two methods were tried.

3.1.4. Ampicillin resistant *E. coli*

T. domestica silk was tested against ampicillin resistant *E. coli*

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	2.09	1.67	20.09569	+
2	2	1.85	7.5	+
3	2.15	2.1	2.325581	+
4	1.95	1.88	3.589744	+
5	1.94	2.06	-6.18557	-
6	2.03	1.98	2.463054	+
7	2	2.17	-8.5	-

Table 11

There is significant evidence to suggest that the ampicillin was reducing the growth of the bacteria on the spider silk. There is not significant evidence that the silk reduces the growth of ampicillin resistant bacteria. This could be due to similar modes of action to ampicillin or it could that the affect was too small to be noticed with a small sample size.

T. domestica	Ampicillin resistant *E. coli*	*T. domestica*	Just Silk
Silk sample lower growth	5	Silk alone with lower growth	6
Silk sample higher growth	2	Silk plus amp with higher growth	0
1 tailed Sign test	0.2265	1 tailed Sign test	0.01565
1 tailed t-test	0.206	1 tailed t-test	0.010

Table 12

3.1.5. Estimating the proportion (%) of contaminated bacteria and the effect on absorbance readings

To calculate what percentage of the growth observed in the liquid broth universal tubes was contaminated bacteria, the universals were prepared as normal, then after 24 hours a 50µl sample was withdrawn from the tube and streaked on a plate of agar. The agar was left 24 hours and then the colonies of each species of bacteria were counted, and the percent that were not the test-microbe calculated.

Extrapolating from this an estimate of what the absorbance figures would be had the bacteria not been present on the silk can be calculated. Using these reduced absorbance figures for cultures incubated with silk shows a more significant effect on bacterial growth.

Sample	% of bacterial growth not tested microbe
1	17
2	34
3	38
4	40

Table 13

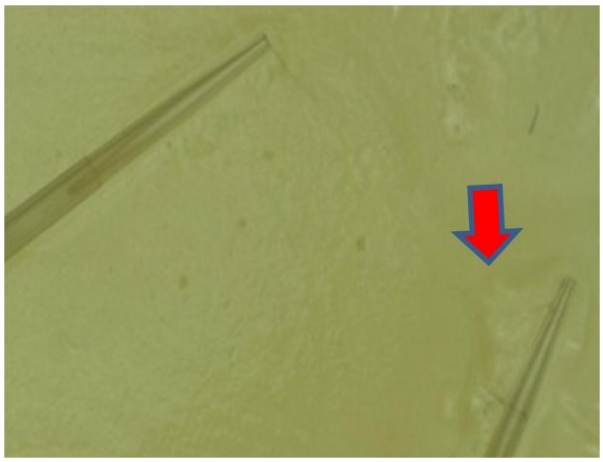
On the 4 samples the average amount of contaminate microbe was 32%. The figures for the silk growth were then reduced by 32% and the t-test comparing them with the control showed that the amount of the tested microbe in the silk samples was significantly lower.

Microbe	1 tailed t-test
B. subtilis	0.00004
E. coli	0.0000007

Table 14

However to conclude from this that silk is inhibiting the growth of both *B. subtilis* and *E. coli* relies on several assumptions about the contaminate bacteria. It assumes that each of the bacteria have an equal affect on the absorbance rate as the test microbes. It also assumes that the contaminate bacteria are not having any effect themselves on the growth of the test microbe. We note, however, that even if contaminating bacteria are not accounted for, our results still indicate a reduction in particular types of microbial growth following incubation with silk.

3.1.6. Agar plate assay



Silk was gathered from the funnel web of *Tegenaria domestica.* Between the two pipettes is one continuous piece of silk. There was a strand of silk between the two pipette tips and a large "clump" of silk at the tip the arrow. Around the clumped area there appears to be a reduction in the amount of bacteria growth. The area of depressed growth very closely mirrors that where the silk is. It is possible that as silk is a solid that the antimicrobial effect does not diffuse out, which is why the area of depressed growth is small.

A second trial with a silk strand. The highlighted area shows where the silk strand was placed. This was a thicker strand that had a drosophila fly wrapped up. The darker dot is where the fly was on the agar. It appears that while there was

bacterial growth from the dead fly, the bacterial growth does not appear to have spread beyond the areas where the silk was laid down.

3.2.1. *Tegenaria duellica*

A small number of trials of a congeneric species were also tested. *T. duellica* lives in same types of environments and spins a funnel web like *T. domestica.*

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.32	0.27	15.625	+

Table 15

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.25	1.25	0	
2	0.74	0.78	-5.40541	-
3	0.066	0.075	-13.6364	-
4	1.75	1.69	3.43	+

Table 16

T. duellica	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	1	1	2
Silk sample higher growth	2	0	2
1 tailed Sign test	1*	1*	1*
1 tailed t-test	0.452	-	0.184

Table 17

The results from such a small data set are inconclusive, but it does appear unlikely that the silk inhibits *E. coli* but that it could inhibit *B. subtilis* in the same way as indicated from *T .domestica*. Based on these results, there does not appear to be evidence to suggest that *T. duellica*'s web silk inhibits the growth of bacteria.

3.3.1. *Atypus* as a bandage

Water absorbance:

The *Atypus* silk initially weighed 0.10 grams. After being removed from the water it weighed 0.29 grams, after blotting to remove surface water droplets it weighed 0.20 grams. This indicates the silk is able to absorb its own weight in water.

Water proofing:

The Eppendorf tube with the silk on top remained dry whereas the tube with the cotton wool did not prevent the water soaking though and was filled with water.

However when the experiment was repeated with roughly 4 times as much cotton wool, the cotton wool did prevent the water soaking though. This indicates that *Atypus* silk is very good at preventing water permeating through.

Microbe proofing:

Soil particles were observed to be stuck to the *Atypus* silk. These were not easily removed despite shaking and centrifuging. Bacteria were observed to grow from the *Atypus* silk making precise observation difficult the size and shape of the colonies did not indicate that these were *E .coli*

Silk from the Mygalomorphae

3.4.1. *Lasiodora parahybana*

10 juvenile spiders were included in this experiment

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below. Trials 1-6 were on silk from individual 1, trials 7-12 from individual 2, trials 13-15 from individual 3, trials 16-17 from individual 4, trials 18-19 from individual 5, trial 20 from individual 6, trails 21-22 from individual 7, trials 23-26 from individual 8, trials 27-32 from individual 9 and trials 33-36 from individual 10.

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	2.21	2.35	-6.33484	-
2	2.11	2.26	-7.109	-
3	2.26	2.5	-10.6195	-
4	2.26	2.14	5.309735	+
5	2.5	2.5	0	-

6	2.49	2.48	0.401606	+
7	2.21	2.33	-5.42986	-
8	2.11	2.2	-4.2654	-
9	2.26	2.09	7.522124	+
10	2.26	2.25	0.442478	+
11	2.5	2.5	0	-
12	2.49	2.41	3.212851	+
13	2.26	2.19	3.097345	+
14	2.26	1.96	13.27434	+
15	2.49	2.5	-0.40161	-
16	2.21	2.33	-5.42986	-
17	2.11	2.16	-2.36967	-
18	2.26	2.5	-10.6195	-
19	2.49	2.42	2.811245	+
20	2.26	2.25	0.442478	+
21	2.21	2.12	4.072398	+
22	2.11	2.31	-9.47867	-
23	2.21	2.31	-4.52489	-
24	2.11	2.33	-10.4265	-
25	2.26	2.47	-9.29204	-
26	2.26	2.29	-1.32743	-
27	2.21	2.22	-0.45249	-
28	2.11	2.08	1.421801	+
29	2.26	2.3	-1.76991	-
30	2.26	2.18	3.539823	+
31	2.5	2.28	8.8	+
32	2.49	2.41	3.212851	+
33	2.21	2.46	-11.3122	-
34	2.11	2.26	-7.109	-
35	2.26	2	11.50442	+
36	2.49	2.07	16.86747	+

Table 18

24 Hours *L. parahybana*	*E. coli*
Silk sample lower growth	16
Silk sample higher growth	18
1 tailed Sign test	0.577
1 tailed t-test	0.642

Table 19

Based on these results, *L. parahybana* spider's silk does not appear to be able to inhibit the growth of bacteria. Although *L. parahybana* builds long lasting webs like *T. domestica*, the two species are not closely related (see figure 1.) The difference in inhibition of bacteria could be explained by the species phylogenetic distance.

3.5.1. Silk from Araneidae

Two species of orb weavers were examined, *Araneus diadematus* and *Zilla diodia*

There were 4 individuals of *A. diadematus,* 3 females and 1 male.

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below. Trial 1 was on silk from individual 1, a female.

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.93	1.98	-2.59	-

Table 20

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below. Trials 1-3 were on silk from individual 1, a female, trial 4 was on individual 2, a female, trials 5-6 were on individual 3, a female, trials 7-8 on individual 4, a male.

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	2.18	2.22	-1.83	-
2	0.74	1.07	-44.59	-
3	0.74	0.71	4.05	-
4	0.74	0.80	-8.10	-
5	0.74	0.77	-4.05	-
6	0.74	1.09	-47.3	+
7	0.74	0.80	-8.10	-
8	0.74	0.66	10.81	+

Table 21

A. diadematus	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	2	0	2
Silk sample higher growth	6	1	7
1 tailed Sign test	0.8555	0.66	0.91
1 tailed t-test	0.93	-	0.946

Table 22

From these results, there is no evidence that *A. diadematus* web silk is inhibiting the growth of bacteria. Greater bacterial growth appeared to occur in the presence of silk. This could be because of the bacteria already present on the silk, or that the bacteria are able to use the silk as a food source. However the latter would be unlikely to have a noticeable affect due to the small volume of the silk relative to the nutrient broth and contamination of silk by other bacteria appears to be the more likely explanation. When the data was split into males and females no difference was observed. *A. diadematus* is fairly closely related to *T. domestica* phylogenetically, but unlike *T. domestica*, *A. diadematus* does not build long lasting webs.

3.5.2. There was 1 individual of *Z. diodia*

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below.

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.93	1.98	-2.59	-
2	0.63	0.43	31.75	+
3	1.22	0.54	55.74	+
4	0.57	0.57	0	
5	1.29	0.40	68.99	+
6	0.32	0.28	12.50	+
7	1.03	1.08	-4.85	-

Table 23

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	.76	.71	6.58	+
2	.89	.90	-1.12	-
3	.94	1.05	-11.70	-
4	1.03	.97	5.83	+
5	2.50	2.45	2.00	+

Table 24

Z. diodia	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	3	4	7
Silk sample higher growth	2	2	4
1 tailed Sign test	1*	0.34	0.26
1 tailed t-test	0.408	0.065	0.061

Table 25

There is possible evidence to suggest that the web silk of *Z. diodia* has an inhibitory affect on the growth of *B. subtilis* (table 22) bacteria, although the findings were not significant. It would have been desirable to have more repeat trials but unfortunately this individual died and as a relatively rare spider in the UK it did not prove possible to obtain another *Z. diodia*.

3.6.1. Linyphiidae

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.63	0.55	12.70	+
2	1.22	0.56	54.10	+

Table 26

The data for trials of silk with *E.coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.76	0.65	14.47	+
2	0.89	0.88	1.12	+

Table 27

There is some evidence that this Linyphiidae's web silk does posses antibacterial properties, however, unfortunately only a limited number of trials took place before the spider died.

Linyphiidae	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	2	2	4
Silk sample higher growth	0	0	0
1 tailed Sign test	0.25	0.25	0.0625
1 tailed t-test	0.221	0.212	0.124

Table 28

3.7.1. Egg silk of *Pityohyphantes phrygianus*

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.59	0.59	-0.85	-
2	0.58	0.54	6.09	+
3	1.54	0.90	41.56	+
4	1.29	0.37	71.32	+

Table 29

The data for trials of silk with *E.coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.88	0.91	-3.41	-
2	0.87	0.90	-3.45	-
3	1.11	0.66	40.54	+
4	0.94	0.92	2.13	+

Table 30

The data for trials of silk with *B. subtilis* incubated in LB broth for 72 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.77	1.30	26.55	+
2	1.80	0.39	78.33	+

Table 31

The data for trials of silk with *E.coli* incubated in LB broth for 72 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.29	1.27	1.55	+
2	2.50	0.95	62.00	+
3	1.06	0.94	11.32	+

Table 32

From the samples gathered, it appears that sometimes the egg silk can have quite a strong inhibitory affect on the bacteria, however it does not appear to happen with all the samples.

Examined after 72 hours (tables 31 and 32), a similar pattern was observed as when examined after 24 hours (tables 29 and 30) – although in the case all the samples showed lower growth. All the samples examined after 72 hours were also examined after 24 hours. One egg silk sample showed higher growth than the control at 24 hours and then lower growth than the control at 72 hours. Of the 5 samples at 72 hours 4 of them showed a greater level of inhibition than they did at 24 hours. It appears that the inhibitory effect of egg silk is maintained for a longer period of time than the web silk of *Tegenaria domestica*.

24 hours Egg silk	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	2	3	5
Silk sample higher growth	2	1	3
1 tailed Sign test	1*	0.312	0.3636
1 tailed t-test	0.221	0.09	0.049

Table 33

72 hours Egg silk	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	3	2	5
Silk sample higher growth	0	0	0
1 tailed Sign test	0.125	0.25	0.03125
1 tailed t-test	0.186	0.148	0.0455

Table 34

3.8.1. Treatment tests to identify the chemical properties of the antimicrobial agent(s)

To potentially identify the compounds on the spider silk that is inhibiting the growth of microbes, various treatments were used on the silk, which was then tested for its antimicrobial activity by the methods described above.

3.8.2 Ultra Violet Treatment

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.32	0.26	18.75	+
2	0.54	0.53	.93	+
3	0.39	0.37	5.13	+
4	0.58	0.55	5.17	+
5	0.71	0.68	4.23	+

Table 35

Ultra Violet Light	*B. subtilis*
Silk sample lower growth	5
Silk sample higher growth	0
1 tailed Sign test	0.0312
1 tailed t-test	0.016

Table 36

There is evidence to suggest that silk subjected to Ultra Violet light is able to inhibit the growth of *B. subtilis*. Based on this data the effect appears to be more pronounced then when the silk is used without being treated by UV light although the difference was not significant, 14 out of 18 trials with lower growth when untreated compared with 5 out of 5 trials with lower growth when treated, Fisher's exact test p = 0.53. A possible reason for this is that the UV light is killing some of the bacteria that appear to be present on the untreated samples.

3.8.3. Freeze Treatment

The data for trials of silk with *B. subtilis* incubated in LB broth for 72 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.32	0.30	6.25	+
2	0.54	0.88	-64.49	-
3	0.58	0.58	0.0	+

Table 37

-20 °C	*B. subtilis*
Silk sample lower growth	1
Silk sample higher growth	1
1 tailed Sign test	1*
1 tailed t-test	0.7715

Table 38

There does not appear to be any evidence that when treated by being frozen spider silk is able to inhibit the growth of bacteria. As untreated spider silk appeared to be inhibiting the growth of *B. subtilis* it is possible that the cold treatment is deactivating the antimicrobial properties.

3.8.4. Heat treatment

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.32	0.88	-175.00	-
2	0.54	0.97	-81.31	-
3	0.58	0.55	5.17	+

Table 39

80 °C	*B. subtilis*
Silk sample lower growth	1
Silk sample higher growth	2
1 tailed Sign test	1*
1 tailed t-test	0.8925

Table 40

There is not significant evidence that spider silk that has been subject to heat treatment is able to inhibit the growth of bacteria. As untreated spider silk

appeared to be inhibiting the growth of *B. subtilis* it is possible that heat treatment is deactivating the antimicrobial properties.

3.8.5. Ethanol treatment

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.58	1.45	-150.00	-
2	0.71	0.64	9.86	+
3	0.61	0.75	-22.95	-
4	0.54	0.65	-21.50	-

Table 41

Ethanol	*B. subtilis*
Silk sample lower growth	1
Silk sample higher growth	3
1 tailed Sign test	0.312
1 tailed t-test	0.8535

Table 42

There does not appear to be any evidence that ethanol treated spider silk is inhibiting the growth of bacteria. Curiously the growth on the samples with silk added was often higher, when it might be expected that the silk would have soaked up some of the ethanol, which does inhibit the growth of bacteria. One possible explanation is that bacteria coating the silk are able to avoid being killed by the ethanol because they are in small spaces within the silk web.

3.8.6. Proteinase K treatment

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.49	0.50	-2.04	-
2	0.50	1.29	-158.00	-
3	0.55	0.51	7.27	+
4	1.16	1.20	-3.45	-

Table 43

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.76	1.86	-5.68	-
2	2.21	2.26	-2.26	-

Table 44

Proteinase K	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	0	1	1
Silk sample higher growth	2	3	5
1 tailed Sign test	0.75	0.68	0.89
1 tailed t-test	0.8975	0.807	0.865

Table 45

It appears from these results that spider silk soaked in Proteinase K is not antimicrobial, this could be because the Proteinase K is denaturing the antimicrobial compounds on the spider silk. This data could also suggest that the antimicrobial compounds are soluble in Proteinase K and the antimicrobial compounds have leeched off into the Proteinase K. 1 out 4 trials with lower growth when treated with Proteinase K compared with 14 out of 18 trials when untreated, using Fisher's exact test, the results were almost significant P = 0.076 that silk treated with Proteinase K has an effect on the silk's inhibition of *B. subtilis*

3.8.7. Proteinase K run off

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.49	0.29	40.82	+
2	0.50	0.42	16.00	+
3	0.55	0.35	36.36	+
4	1.16	1.10	5.17	+

Table 46

Proteinase K Run-off	*B. subtilis*
Silk sample lower growth	4
Silk sample higher growth	0
1 tailed Sign test	0.0625
1 tailed t-test	0.0185

Table 47

There is significant evidence that Proteinase K, which has had spider silk soaking in it, can inhibit the growth of bacteria. However this could be just the Proteinase K that is having the inhibitory affect, so a control of just Proteinase K was used.

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.44	0.29	40.82	+
2	0.74	0.42	16.00	+
3	0.38	0.35	36.36	+
4	0.62	1.10	-77.00	-

Table 48

Proteinase K Run-off compared with control Proteinase K	*B. subtilis*
Silk sample lower growth	3
Silk sample higher growth	1
1 tailed Sign test	0.312
1 tailed t-test	0.4895

Table 49

There is no evidence to suggest that Proteinase K that has had spider silk soaking in it has a greater inhibitory affect on bacteria than Proteinase K alone fishers exact test p=0.5. From this data it would not appear that the antimicrobial compounds on the silk are soluble in Proteinase K. As Proteinase K denatures protein this could indicate that the antimicrobial compounds are Proteins that have been denatured.

3.8.8. Water Soak

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.61	0.55	9.84	+
2	0.54	1.00	-86.92	-
3	0.71	0.65	8.45	+
4	1.10	1.06	3.64	+
5	0.42	0.53	-26.19	-
6	0.42	0.49	-16.67	-
7	0.49	0.51	-4.08	-

Table 50

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.86	0.88	-2.33	-
2	0.86	0.89	-3.49	-
3	0.86	0.84	2.33	+
4	1.44	0.96	33.33	+
5	1.44	0.93	35.42	+
6	1.44	0.92	36.11	+
7	0.98	1.01	-3.06	-
8	0.98	0.92	6.12	+
9	0.98	1.05	-7.14	-

Table 51

Water soak	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	5	4	9
Silk sample higher growth	4	3	7
1 tailed Sign test	1*	1*	0.402
1 tailed t-test	0.050	0.8285	0.185

Table 52

There is not significant evidence to suggest that spider silk soaked in water is able to inhibit the growth of bacteria. This appears to indicate that the antimicrobial compounds on the silk are either denatured by water or that they are soluble and leeched off in the water.

3.8.9. Water Run off

The data for trials of silk with *B. subtilis* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	0.48	0.61	-27.08	-
2	0.44	0.30	31.82	-
3	0.56	0.56	0.0	+
4	0.45	0.49	-8.89	-

Table 53

The data for trials of silk with *E. coli* incubated in LB broth for 24 hours are given below

Trial No.	Control Absorbance Reading	Silk Sample Reading	Inhibition %	Sign test
1	1.57	1.74	-10.83	-
2	2.17	2.12	2.30	+

Table 54

Water run off	*E. coli*	*B. subtilis*	All bacteria
Silk sample lower growth	1	2	3
Silk sample higher growth	1	1	2
1 tailed Sign test	1*	1*	1*
1 tailed t-test	0.341	0.451	0.308

Table 55

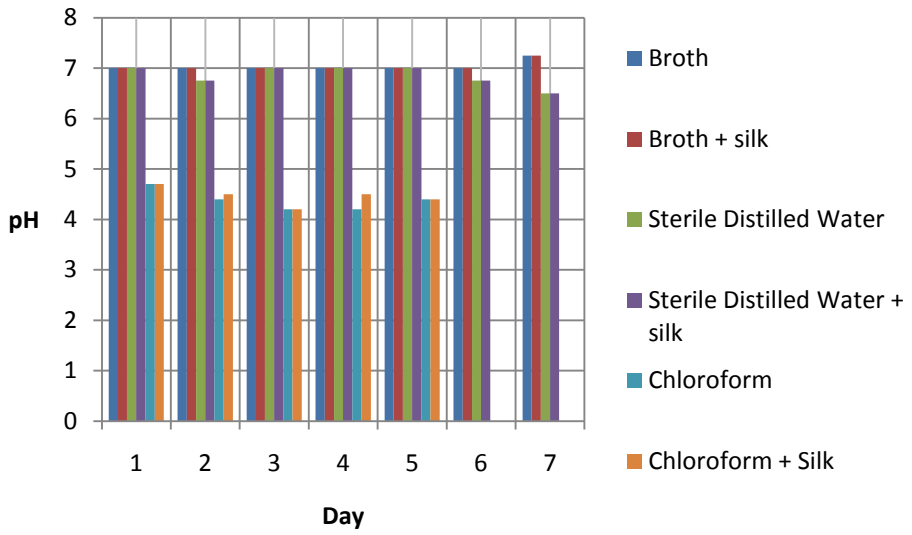
When water that has spider silk soaked in it is compared with the control water, the evidence does not suggest that the growth of microbes is significantly lower. As the silk did not show antimicrobial activity after being soaked in water but neither did the silk with water soaked in it, this indicates that possibly the antimicrobial compounds are either denatured in water or that insufficient amounts were leeched into the water.

3.9.1. pH effect of spider silk

There are some suggestions that the reason spider silk is not readily decomposed by microorganisms is because the silk is highly acidic (Heimer 1988). To test if it was just the acidity of the silk that was inhibiting the growth of the bacteria, an experiment was set up examining if the silk would turn sterile distilled water or liquid broth more acidic. The paired samples of the silk and control were placed on a shaker at 170 rpm 30 °C. Every 24 hours a pH indicating strip was inserted into the liquid and the pH level recorded. The test was also done using chloroform instead of liquid broth or water.

Medium	Sample	pH Day 1	pH Day 2	pH Day 3	pH Day 4	pH Day 5	pH Day 6	pH Day 7	pH Day 8
Broth	Silk	7	7	7	7	7	7	7	7.5
Broth	Control	7	7	7	7	7	7	7	7.5
Sterile Water	Silk	7	6.5	7	7	7	7	6.5	6
Sterile Water	Control	7	6.5	7	7	7	7	6.5	6
Broth	Silk	7	7	7	7	7	7	7	7
Broth	Control	7	7	7	7	7	7	7	7
Sterile Water	Silk	7	7	7	7	7	7	7	7
Sterile Water	Control	7	7	7	7	7	7	7	7
Chloroform	Silk	4.7	4.5	4.2	4.5	4.4	-	-	-
Chloroform	Control	4.7	4.4	4.2	4.2	4.4	-	-	-

Table 56



The pH of the silk and control samples was subject to the paired samples t-test to see if there was any difference in the samples

The figures were analysed and there is not significant evidence to suggest that the presence of silk affects the pH of the media tested. P = 0.324

The three media were also analysed separately and there was no evidence that the pH of either liquid broth, sterile distilled water, or chloroform was affected by the presence of spider silk. P = 0.374

3.10.1. Examining the effect of spider silk on mammal cells

Tegenaria domestica appears from our data to show the strongest antimicrobial affect of all the silks tested. By showing the strongest antimicrobial affect this silk is both the most likely to have potential health benefits and the most likely to have a negative effect on mammal cells. It was therefore chosen as the subject to test against a mammalian cell line.

Date Of Trial	Number of Million Control Cells per ML	Number of Million Silk cells per ML	Control pH 1 day	Silk pH 1 day	Control pH 1 week	Silk pH 1 week
16/09/2010	1.12	1.08	6.4	7	7.4	6.5
21/20/10	0.72	0.54	6.7	6.7	6.7	6.7
23/10/2010	0.44	0.52	7.2	6.7	7.2	6.7
24/10/2010	0.4	0.54	7.2	7.2	7.2	7.2

Table 57

Using the paired samples t-test it was found there was no significant difference in the silk and control samples number of mammal cells P = 1.000.The t-test did not find significant difference in the pH of the samples. Evidence that the presence of silk effects the pH (alkaline) after 1 day P = 0.919. Evidence that silk affects the pH (acid) after 1 week 0.207

The results show that there is no significant difference in the growth of mammal cells whether in the presence of spider silk or not. There is some slight, but non significant evidence that the presence of spider silk has some effect on the pH. The silk samples were slightly more acidic.

Discussion

4.1.1. Antimicrobial activity of *Tegenaria domestica* silk

The possibility that spider silk has therapeutic applications through antimicrobial activity has been raised by previous authors (Heimer 1988; Vollrath, Fairbrother et al. 1990) but until now there have been few studies to test this hypothesis. In this study we demonstrate that some silk types likely do inhibit bacterial growth, although only certain types of bacteria may be susceptible. The strongest evidence supporting the view that silk has antibacterial properties comes from work on silk from *Tegenaria domestica*, the common house spider. This species belongs to the Agelenidae and uses its silk for prey wrapping and constructing funnel webs.

The studies appear to show that silk of spiders such as *T. domestica* may have antimicrobial properties. There appears to be activity against gram positive *B. subtilis*, although the effect does not appear long lasting and 48 hours after the start of the experiment, no activity against microbes can be detected. There appears to be no similar effect on the gram negative *E. coli* or against fungi, although it may be that effects on fungi were undetected in our experiment.

There was a large degree of variation in bacterial growth following incubation with silk, likely because the silk itself carries bacteria. The variation could also be explained by the different amounts of silk added to the trials. However it was observed anecdotally that the amount of silk added did not correlate with the amount of inhibition. Further tests demonstrated that the number of bacteria colonising the silk, which were potentially confounding our results on inhibition of laboratory test bacteria, was large. E.g. up to 40% of those bacteria found in an incubated sample after 24 hours.

The presence of these additional bacteria means that our results are likely to be conservative and the conclusions remain robust.

However the high amount of variation in the data meant that the inhibitory affect of the silk was not shown on every single sample. When examined after 24 hours of growth there was a trend in the samples with silk present showing less growth of *E. coli* and *B. subtilis*. Additionally there was a large amount of variation in the amount of growth in the samples with silk present. A possible explanation for this is that there are bacteria already present on the silk which also grow in the liquid broth, leading to an increase in absorbance figures. Tests where the silk was inserted into the liquid broth alone would generally show that bacteria from the silk would grow. These bacteria could either be present on particles, for example soil, stuck to the silk, or they could exist on the silk itself.

While there was a trend for the silk to inhibit both bacterial types, the difference in growth was only significant against individual species of bacteria when the growth of the bacteria *B. subtilis* was tested, possibly because the occasions when the silk samples showed higher growth there was often much higher growth. This contrasts with when the silk sample showed lower growth, the difference in growth was smaller. It is possible that silk is more effective against *B. subtilis*, so the reduction in growth would require a higher level of other bacteria to be "masked". It is also possible that the bacteria that are present on the silk are inhibiting the growth of the *B. subtilis* or that *B. subtilis* is inhibiting the growth of the bacteria also present on the silk. The results showing significant inhibition against *B. subtilis* but not *E. coli* suggests that the silk is effective against a gram positive bacteria (*B. subtilis*) but not a gram negative bacteria (*E. coli*). It appears that spider silk is a narrow spectrum antibiotic like many antimicrobials for example (Perry, Blunt et al. 1991; Yamamoto, Kitayama et al. 2001; Lai, Huck et al. 2002).

It is interesting to note that *B. subtilis* is a soil bacteria unlike *E. coli* (Henschke and Schmidt 1989). This could explain why the silk had an effect on *B. subtilis* but not *E. coli*. If the silk, regularly being built around soil, is much more likely to encounter *B. subtilis* then there will be stronger evolutionary pressure for the silk to be able to inhibit the growth of *B. subtilis* than *E. coli*.

T. domestica is a funnel web spider that builds a long lasting web (Roberts 1995). As the spider builds the web to be long lasting it would be beneficial to the spider if the web was able to resist microbial decomposition. If the web is able to resist microbial decomposition then it means that the spider will be allocating fewer resources to web manufacture, provided that the cost of making the web antimicrobial does not exceed the cost of having to rebuild the web more frequently. Another possible benefit of the web being antimicrobial is that as the spider resides on the silk, then the spider will be less likely to come into contact with microbes that could have a negative effect on the health of the spider.

The *T. domestica* samples were also examined after 48 hours of growth. While the samples with silk present showed lower growth the majority of times, the difference was not significant. The data suggests that the inhibitory effect of the silk reduces with time. A possible explanation for this that the antimicrobial effect of the silk wears off after a short period of time. However this would appear unlikely because the silk would often be gathered after it was several weeks old, and still show an antimicrobial effect in the first 24 hours. Also it has been anecdotally observed that spider webs can often remain in nature for years and are known for their high durability (Vollrath and Porter 2006), so it seems unlikely that the antimicrobial affect is only short lived on the web. It could be in the presence of the broth the silk is altering and so after a period of time the antimicrobial effect is reduced.

Another possible explanation is that after a period of 48 hours, the silk is no longer the primary limiting factor in the growth of the bacteria. If the bacterial growth is reduced by a limiting factor that is affecting the growth rate more than the presence of the silk, then the effect of the silk will not be shown as strongly. A likely limiting factor that would increase with time would be resources, it is possible that the samples without silk have quicker reached the stage where resources are the limiting factor and so the lack of resources slows down the growth. Compared with the samples with silk which have lower growth after 24 hours which would still have sufficient recourses and so would catch up the samples without silk (Hershey and Bronfenbrenner 1938).

It is also possible that silk is bacteriostatic rather than bactericidal. If the silk was bacteriostatic it would mean the silk is having a slowing effect on the growth of the bacteria, rather than if it was bactericidal, which kills the bacteria. Bacteriostatic action against *B. subtilis* by antimicrobials is well documented (Ogilvie, Wiebauer et al. 1975; Bonvehí, Coll et al. 1994; Kubo, Fujita et al. 2004) and it is possible the silk is operating in this manner. A possible explanation for the observation of the bacteria being present on the silk yet the silk being resistant to decomposition could be that once the bacteria come into contact with the silk they stop growing because of its bacteriostatic effect, but once placed in the liquid broth they come loose from the silk and so are free from its bacteriostatic affect. The bacteria, which were not killed by the silk, would then show growth in the liquid broth.

Experiments were done using ampicillin resistant *E. coli*. Ampicillin was added to both the silk and control samples with the rational that it should kill the bacteria on the silk but not affect the *E. coli*. The results showed a trend towards the silk samples having lower growth. The trend towards the silk samples having lower growth was slightly stronger than the trend of silk samples to have lower growth with non-ampicillin resistant *E. coli*. However only low numbers of samples were

tested and the results did not show a significant difference at $P < 0.05$. A Possible explanation is that the bacteria present on the silk are also resistant to ampicillin. Also the ampicillin resistant *E. coli* may be resistant to spider silk. This may be caused by a similar mode of action of ampicillin and spider silk. The silk was tested with ampicillin but no ampicillin *E. coli* present. There was still sometimes growth on the silk and ampicillin samples. This indicates that at least sometimes there are ampicillin resistant bacteria present with the silk. It is also a possibility that the ampicillin interfered with the silk, leading to a reduction in the antimicrobial affect. It is worth noting that ampicillin is a β-Lactam antibiotic, it is possible that silk has a similar mode of action to β-Lactam antibiotics, of which some, like penicillin are ineffective against gram negative bacteria and fungi (Berkow, Beers et al. 2008). Additionally as resistance to ampicillin is conferred by β-Lactamase (Robicsek A 2006), this could mean that the ampicillin resistant *E. coli* is also resistant to *T. domestica* silk, if it has a similar mode of action to ampicillin.

As stated above there were lots of bacteria present on the silk that would also grow in the sample. Taking samples of the growth of the silk and the bacteria from the universal tubes, which would be spread on agar plates, it could be see what percentage of the growth in the tubes was not down to the tested microbe. Examining the plates revealed that on average 32% of the bacteria growing were not the tested microbe, and even on the agar plate with the greatest percentage of *E. coli* colonies showed 17% non *E. coli* growth. Assuming most samples fall within this range, then in the samples with silk present the level of the tested microbe would be lower than what was estimated by the absorbance figures. If the amount of non tested microbe is 32% then there is less growth in the *B. subtilis* samples with silk at significance of $p < 0.00005$, in the *E. coli* samples there is less growth with significance of $p < 0.000001$. This suggests that both a gram positive and a gram negative bacteria are inhibited by spider silk. However this does rely on the assumption that if the other bacteria were to be removed, that this wouldn't affect

the growth of the tested microbe at all. As there is competition for resources this would appear to be unlikely that the removal of the other bacteria would not increase the growth of the tested microbe. However the presence of other bacteria that are growing in the liquid broth would that the growth levels of the tested microbe are lower than calculated by the absorbance assay.

T. domestica silk was tested against two species of fungi, *S. cerevisiae* and *A. niger*. While *S. cerevisiae* showed growth evenly distributed in the universal tube and therefore was suitable for the growth to be tested via light absorbance, *A. niger* would grow in clumps making it unsuitable for growth to be examined via this method. Possible assays that could assess the growth of *A. niger* could involve drying the growth medium from the universal then weighing the *A .niger*, however the differences in weight would likely only be small so highly accurate equipment would be required. Also it might be possible to reduce the clumping by having the growth period of *A. niger* on a high speed rotation. *S. cerevisiae* showed higher growth with silk present an equal number of times to when the control sample had higher growth. The average growth of the samples with silk present was lower, but not significantly. It is possible that the antimicrobial effect of spider silk is limited to bacteria, and that the silk does not inhibit the growth on fungi. It is also possible that as with the bacterial samples, the growth of bacteria present in the silk is "masking" the effect the silk is having on the fungi. Another possibility is that spider silk does possess antifungal properties, but that laboratory *S. cerevisiae* is either resistant to or unaffected by it. It would be unlikely that the benefits conferred by being antibacterial would not also be conferred by having antifungal properties, and anecdotal evidence would suggest that cobwebs (spider webs no longer occupied) often are not decomposed by either bacteria or fungi.

A different species of *Tegenaria, T. duellica* was also tested however the data did not appear to be evidence of antimicrobial activity. This could be explained by the

small sample size or it could also be that the antimicrobial activity is only found in a certain species of *Tegenaria*. *T. duellica* lives in similar environments to *T. domestica* so it would possibly be expected to have similar antimicrobial activity, however it was observed anecdotally that the silk of *T. duellica* had more particles present on it. It is possible that these particles contained bacteria and this produced a greater "masking" effect of the silks effect on the test microbe.

4.1.2. Treatment tests

To identify the compounds on the spider silk that are having the antimicrobial properties the silk was subject to various treatments. Together with inactivation by Proteinase K, tests showing that antimicrobial activity is heat and freeze inactivated indicate that the active agent may be a protein that is not susceptible to the level of UV irradiation applied.

After being soaked in Proteinase K for 1 hour the silk was examined to see if it had antimicrobial properties. A possible explanation for this is that the antimicrobial compounds present is a protein and so when soaked in Proteinase K the compounds are denatured (Betzel, Singh et al. 1993). Another possibility is that that the antimicrobial compounds are not denatured by the Proteinase K but it is soluble in it and so is leeched off into the Proteinase K.

The Proteinase K that had spider silk soaking in it was tested to examine the possibility that the antimicrobial compounds present on the spider silk. There was a trend towards the Proteinase K that had had spider silk present showing lower bacterial growth than the control sample, but the difference was not significant. A possible explanation for this is that the antimicrobial compounds from the silk had leeched off into the Proteinase K, which then would inhibit the growth of the bacteria.

The silk was subject to 20 minutes of ultra violet radiation. This had two purposes, first it would examine if the antimicrobial property would stop after the silk had been subject to ultra violet radiation, secondly the UV rays would kill some of the bacteria present on the silk (Villarino, Bouvet et al. 2000), which meant that it could be tested if silk with less bacteria present would show a stronger antimicrobial affect. When treated with UV the silk reduced the growth of the microbe at a significance of $p < 0.05$ using the t-test and was very nearly significant using the sign test. This indicates that subjecting the silk to UV does not affect its antimicrobial property. As spider silk is subject to UV in the environment it is to be expected that the antimicrobial property is not removed by exposure to UV rays. However as the spider examined was a funnel web spider it could be argued that as they build their webs in corners they are often not heavily exposed to sunlight. Additionally as the silk treated with UV showed a more consistent and stronger reduction in growth of the test microbe than untreated silk it is possible that because the UV killed some of the bacteria present on the silk, then this reduced the affect that the other bacteria where having on the absorbance rates. This might show a "truer" representation of the effect that spider silk has on the tested microbes.

The spider silk was also frozen at minus 20°C for 1 hour. This is a colder temperature than *T. domestica* would experience naturally. The results appear to indicate that spider silk is no longer antimicrobial after being frozen. This could be because freezing of the silk destroys the antimicrobial property. As this is a colder temperature than would likely be experienced naturally, then if the silk maintained its antimicrobial properties at very low temperatures would not confer advantages to the spider.

After being subject to 80°C for 1 hour the spider silk did not appear to posses antimicrobial properties. It is possible that the heat would lead to denaturing of the antimicrobial compounds which would mean bacterial growth would no longer be inhibited. As this temperature is far out of the range a spider's web would normally be exposed to there would not be an advantage to a spider making their web antimicrobial even in extreme heat. However it would be expected that being subject to this heat should also kill the bacteria present on the silk, but unlike when the silk was subject to UV treatment, the silk treated with heat appeared less inhibitory than untreated silk. It is possible that the silk, having been heated and lost its antimicrobial defence, would then serve as nutrients for the bacteria when placed in the broth. However as the silk has very little mass compared with the mass of the broth this is unlikely. As there was not significantly more growth in the samples with silk present it is likely that more growth was shown in these samples because of chance.

The silk was treated by being soaked in ethanol for 1 hour then its antimicrobial activity was examined. This data does not indicate that after being soaked in ethanol silk has antimicrobial properties. This could be because the ethanol is denaturing the antimicrobial compound (Piper 1995). Interestingly after being soaked in ethanol the sample with spider silk present tended to show increased growth. It would be expected that that after being removed from the ethanol, the spider silk would contain traces of ethanol. As ethanol is known to inhibit microbial growth (Rigomier, Bohin et al. 1980; Ingram 1989) it could be expected that the samples would show a slight decrease in growth. Additionally the ethanol would possibly kill some of the bacteria present on the silk. However the growth was not observed as being slower. It is possible that only trace amounts of ethanol had remained on the spider silk and so the effect was too slight to be detected by this assay. Also the exposure time to ethanol of 1 hour could be too short to have a significant detrimental effect on the bacteria.

The spider silk was soaked in water for 1 hour to see if this would affect the antimicrobial properties. There was a trend for the spider silk samples to show lower growth, but the difference was not significant. However when just the affect on *E. coli* is examined using a 1 tailed t-test there is lower growth on the samples with silk added at a significance of $P \leq 0.05$. The data did not suggest that silk soaked in water has an inhibitory effect on the growth of *B. subtilis*. This could be explained by the silk's antimicrobial mode of action being different for *E. coli* and *B. subtilis*, and that when subject to being soaked in water, the antimicrobial compound loses its ability to inhibit *B. subtilis*. As spider silk in nature is subject to frequently being wetted by rain, it would be expected that the antimicrobial properties would be maintained after the silk. However it is possible that the silk only shows a reduction of its antimicrobial property after being saturated in water, rather than subject to rain. Another possibility is that when in contact with water the silk gradually loses the antimicrobial compounds, with this results it could explain why there was still a trend towards the silk showing inhibition of growth of bacteria, but the trend was weaker when compared against silk that had not been soaked in water. It is possible that the antimicrobial compounds of the silk are soluble, and that it is leeching into the water.

To test if the antimicrobial compounds had leeched into the water, the water that had silk soaked in it was added to the bacteria growing and an equal amount of water added was used as the control. There was a trend for the water that had had silk soaked in to lower the growth of bacteria but the trend was not significant. As discussed above, it is possible that there is a limited leeching of the antimicrobial compounds into the water, this would explain why both the silk soaked in water and the water that had silk soaked in it still showed a trend towards antimicrobial activity, but that the trend was weaker when compared with silk that that was not soaked in water.

Heimer (1988) stated that the reason spider silk is able to resist decomposition is because spider silk is acidic and so bacteria are unable to thrive. To test if it was the spider silk's acid that was affecting the ability of the bacteria to grow, it was seen if the presence of spider silk would lower the pH of the growth media. It was also tested if spider silk would alter the pH of Chloroform and sterile distilled water. The data does not suggest that the presence of spider silk has an effect on the pH of liquid broth. The inhibition of bacterial growth observed using the *T. domestica* silk cannot be explained by the spider silk causing the broth to be become acid and inhibiting bacterial growth. Additionally the data suggest that the presence of spider silk does not affect the pH of Chloroform or sterile distilled water. This is not to suggest that spider silk is not acid, but to suggest that the data suggest that the bacterial growth was not inhibited by the spider silk lowering the pH of the liquid broth.

4.1.3. Antimicrobial activity of silk from other species of spider

Egg silk of *P. phrygianus* was examined to see if it had antimicrobial properties. After 24 hours there was a trend towards the samples with the egg silks showing antimicrobial activity but the trend was only significant when the growth was compared against both *E. coli* and *B. subtilis* with a 1 tailed t-test. Because of the difficultly of obtaining many samples there was not enough to repeat the experiment to see if the reduction in growth would be significant for both individual species of bacteria. The egg silk samples were also examined after 72 hours and there was a trend of the samples having lower bacterial growth, but this was only significant at $P < 0.05$ when the growth of both bacteria were examined using the 1 tailed t-test. The reduction in growth was very nearly significant when the growth of the bacteria was examined using the sign test. Egg silk being antimicrobial would confer benefits to the spider as the spider places a large investment of resources in

the egg and so there would be a benefit for the spider if it can prevent the egg being consumed by microbes. Also the data did not suggest a weakening of the antimicrobial effect with time as was observed with the *T. domestica* web silk. It is possible that the egg silk has a longer lasting or stronger antimicrobial effect than the web silk.

The silk of *L. parahybana* was examined for antimicrobial properties. *L. parahybana* builds long lasting webs but the data did not suggest the webs have antimicrobial properties. This could be because the silk lacks antimicrobial properties or is could be because of the soil present on the silk that the growth of other bacteria are masking the inhibitory effect of the tested microbe. While *L. parahybana* builds long lasting webs the spider is not a close relative of *T. domestica* (see figure 1). This could suggest that the antimicrobial properties of *T. domestica* silk evolved after the split from the Mygalomorphs.

A. diadematus builds new webs daily and so there would be fewer benefits conferred to the spider if it manufactured webs with antimicrobial properties as the spider is devoting resources to rebuilding the web regardless of it the web has decomposed or not. It is also possible that as the spider eats its web daily, then the presence of microbes on the web provide nutrients for the spider when the web is eaten. The silk lacking antimicrobial properties would mean greater amounts of bacteria present on the web and nutrients taken in when the web was ingested by the spider. The data did not suggest that *A. diadematus* silk inhibited the growth of bacteria. There is significant evidence to suggest that the addition of *A. diadematus* silk increased the growth of bacteria.

It is interesting to note while *A. diadematus* silk increased the growth of bacteria 7 times out of 9, the *L. parahybana* silk only increased the growth of the bacteria eighteen times out of thirty four. It is possible that *L. parahybana* silk does posses

some antimicrobial activity, but that this is being covered up by contaminates on the silk. This contrasts with *A. diadematus* silk, which was shown to significantly increase the growth of the bacteria. As *L. parahybana* builds long lasting webs, this would support the hypothesis that long lasting webs are more likely to negatively affect bacteria growth.

Z. diodia is also an orb-weaver that builds new webs daily. There was a trend that the addition of *Z. diodia* silk inhibited the growth of bacteria but the result was not significant. When both bacteria were examined using the 1 tailed t-test there was almost significant evidence that the presence of *Z. diodia* silk inhibits the growth of bacteria.

A linyphiid spider which builds long lasting sheet webs was examined to see if the silk was antimicrobial. There was a trend for the silk to inhibit the growth of bacteria but the findings were not significant. Like the *T. domestica* having antimicrobial silk would confer benefits for the spider.

4.1.4. Potential for therapeutic applications

The *T. domestica* silk was tested to see if it had an effect on the growth of mammal cells. The data suggest there is no evidence that the presence of spider silk inhibits the growth of mammal cells. As spider silk is not naturally exposed to mammal cells there would not be an advantage conferred on the spider if its silk inhibited mammal cells. Snail slime which has been found to be antimicrobial has also been found to not effect mammal cells (Tincu and Taylor 2004; Adikwu and Ikejiuba 2005). But many antimicrobial compounds also have a negative effect on mammal cells so are not useful for medical applications (Helmerhorst, Reijnders et al. 1999; Stallmann, Faber et al. 2005). If the antimicrobial properties of spider silk were used in mammal cells it appears that the mammal cells would not be negatively

affected. While it is not known how much of the antimicrobial agent was introduced to the mammal cells it is known that it was at concentrations that are able to affect bacteria.

4.1.5. *Atypus* silk as a bandage

Heimer (1988) documented that there is a tradition of peasants using *Atypus* spider silk as a bandage and wound dressing. To see if *Atypus* silk would make a good bandage, *Atypus* spider silk was tested to see if it was water proof and it was found that even a thin film of *Atypus* silk would provide a waterproof layer. Even when saturated with water for 24 hours no water soaked though underneath the silk. As it is important for wounds to remain dry to facilitated healing being water proof is highly beneficial in a bandage. The *Atypus* silk was also tested to measure how much water it would soak up if left in water. As discussed above this would maintain a dry environment around the wound, which would help make the area around the wound less ideal for bacterial growth. *Atypus* silk doubled in weight after being soaked in water. However the *Atypus* silk was thickly covered in soil which was difficult to remove. When the *Atypus* silk was placed on solid agar it was observed that there was strong bacterial growth from the *Atypus* silk. This suggests that the silk has high numbers of bacteria present and this would not make it suitable to cover a wound with. It is possible however that the bacteria present on the silk are not harmful to humans and because of the water proofing the *Atypus* silk as a bandage would still reduce the number of harmful bacteria entering the wound.

Future work

5.1.1. Developments from this research

An obvious expansion of this work would be to look at a greater variety of spiders and microbes and see if the antimicrobial properties have evolved just once or have evolved many times and if there are many components or whether the antimicrobial properties are conferred by a single agent.

A more comprehensive breakdown of the testing the different individual silks of a particular spider could be worthwhile future research. Additionally the silk could be compared with how fresh it is, do more recently laid silks show more antimicrobial properties? Another factor of the silk with potential for investigation is the quantity of the silk used in trials. While anecdotal observations undertaken did not appear to indicate a strong correlation between the quantity of silk and the level of inhibition, it is possible other factors where masking the impact quantity had on the level of inhibition.

Possible further study could be examining the bacteria that did already appear to be present on the silk. Are the bacteria present on the silk gram positive or gram negative? Did they decompose the silk or did they survive off other nutrients that were stuck to the silk? Also the amount of the bacteria on cobwebs (spider webs that are no long occupied) could be compared with spider webs, are there more or less bacteria on long abandoned webs? It would be interesting to see if cobwebs tended come from certain species of spiders more than others.

While briefly touched on in this thesis, a more definite and precise identification of the particular molecule of the spider silk that has the antimicrobial properties would

be desirable. Possibly subjecting the spider silk to either mass-spec or High-performance liquid chromatography would be useful in breaking down the spider silk into individual molecules, which could then be individually tested. If the individual compound was identified then the potential medical applications could be researched in depth.

While is it well established that the silkworm moth, *Halophora cecropia* has antimicrobial compounds present, the silk itself has not been widely tested (Boman, Nilssonf.I et al. 1974), as the silk silkworms uses silk to make a cocoon which it resides inside. The benefits to the organism of the silk having antimicrobial properties would be similar to the benefits of the spider's egg silk being antimicrobial, so this would be an interesting future avenue to explore. In addition to the silkworm, other silk using insects could be for the antimicrobial properties of their silk.

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