Project Report

Manuka Honey Gel as a Viable Topical Treatment to decrease Contaminated Equine Distal Limb Wound Healing Times

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Index

Abstract II

Acknowledgements II

Literature Review 1

Factors Affecting the Healing of Equine Distal Limb Wounds

Manuka Honey's Activity

Treatment of Equine Distal Limb Wounds with Manuka Honey

Report 3

Scientific Research Question

Objective

Scientific Hypotheses

Null Hypothesis (H_0)

Hypothesis (H_1)

Associated Risks

Risks Associated with the Handling of Horses

Risks related to Contamination

Proposed Protocol Supporting Methodology

Assumptions Made

Study Admission Criteria

Proposed Methodology and Apparatus

Data Collection and Treatment upon admission

Continuing Data Collection and Treatment

Expected Results from Data Analysis

Discussion

Conclusion

References & Appendices 9

References

Appendices

Abstract

Distal limb wounds make up 85% of equine wound cases. Due to their difficult positioning and prevalence, they place a significant welfare and economic burden on the sport horse and racing industries. Research into their treatment and management is hence highly relevant and important. Their healing is known to be affected by a wide range of factors, including bacterial contamination. As of late, antibiotic resistance incidence has increased, and so a plethora of natural remedies have been actively sought out for reexamination. Honey, a supersaturated sugary solution which has for over 8000 years been administered in medicine, is amongst these. Specifically, the *Leptospermum* honey, Manuka, has been in the focus of this research for its methylglyoxal, non-peroxide antibacterial activity, shown to combat many common bacterial isolates found in the equine environment. To investigate its use as a topical wound treatment, researchers have previously performed small scale studies on surgically created wounds in controlled scenarios. This report proposes a methodology and protocol to be implemented as a multi-centre study to decrease the healing times of more realistic contaminated equine distal limb wounds using Manuka Honey gel. Based on pre-existing relevant data it predicts that via the implementation of these procedures, significant reductions in healing times could be achieved for distal limb wounds.

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Literature Review

Equine distal limb wounds (EDLWs) place a large burden on equine industries due to their treatment often being time-consuming, difficult and expensive, and due to their high incidence rate [62]. The healing of EDLWs is often compromised by a variety of factors, including bacterial contamination, which negatively affect the second-intention healing processes required. Common antibiotics are becoming progressively less effective due to rising incidence of bacterial resistance. This increase in resistance has led to the 'rediscovery' of honey as a health agent [75].

The healing activity of honey lends itself to its anti-bacterial and anti-inflammatory properties [74]. *Leptospermum*, especially Manuka, honeys are being researched vigorously for their suspected benefits. Research investigating Manuka Honey's implementation as a topical treatment for EDLWs has previously only been small-scale and provides conflicting evidence on its efficacy. Comparatively in vitro research has conclusively supported Manuka's ability to combat certain bacterial isolates.

Factors Affecting the Healing of Equine Distal Limb Wounds & Biofilms

Wounds are amongst the most common reasons for an equine's admittance to a veterinary clinic. Most frequently, in 85% of cases, these are located on the distal limb [62]. This being the limb located below the knee or hock of the equine (*Figure 1*).

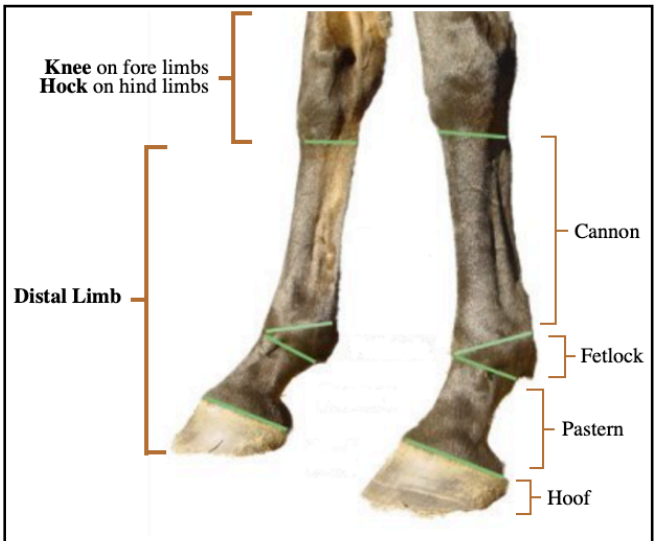

Figure 1: Diagram of Distal Limb on an Equine, from The Distal Limb Bones of the Equine (2020), *Anatomy-of-the-Equine.com*, Available at: http://www.anatomy-of-the-equine.com/distal-limb-bones.html

Wounds in this area are rarely exposed to the conditions needed for ideal closure due to a variety of internal and external factors. A 2010 study [30] described two categories of factors that may affect this process: local (oxygenation, contamination) and systemic factors (age, gender, disease, obesity, etc). In equines, especially local factors impact healing, as environments aren't controllable. Hence, they are forced to heal by second-intention (*Figure 2*). These are often characterised by severe tissue loss, exuberant granulation tissue, gross contamination and a weak inflammatory response [70, 27]. Additionally, bacterial biofilms have been linked to EDLWs. The first study to provide evidence of biofilms in horse wounds was published in 2009 [18]. The following 2011 study followed a very similar method [66] and provided evidence of biofilms in 61.5% of equine wounds and outlined a total 340 bacterial species. Overall, the most prevalent species highlighted were in coherence with the former study, the most prevalent being the *Staphylococcus* genus. As biofilms have been linked to inherent anti-microbial/bacterial resistance and persistent mammalian infections [21], they help explain the slow healing of EDLWs. Nonetheless, it is well documented that the culture of swabbed isolates, the primary method used by both studies, leads to an underrepresentation of species that reside deeper in the wound tissue and an overrepresentation of surface bacteria, such as *Staphylococcus aureus* [25]. More research is hence required to solidify these findings. The aforementioned factors should be considered when trialing new therapies.

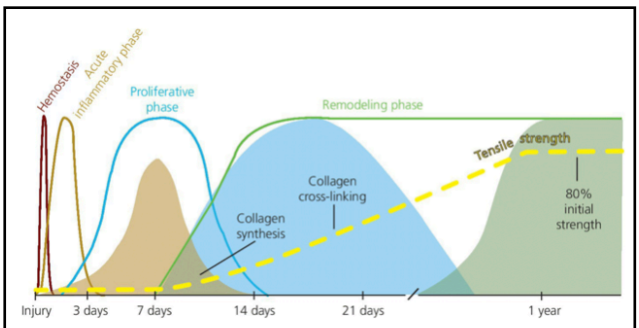

Figure 2: A temporal profile of the phases of wounds healing by second-intention showing the tensile strength of the affected area, modified by Marco Langlois for the Faculty of Veterinary Medicine, University of Montréal from Stashak & Theoret, 2014.

Manuka Honey's Activity

Manuka honey has in recently been pinpointed for its anti-bacterial and anti-microbial activity [45]. It has been shown to disrupt or prevent biofilm

formation [33], which is notable as it is established that biofilms can greatly impact the healing of distal limb wounds [18]. This action is linked to the high methylglyoxal (MGO) content of the honey, as honeys infused with MGO have a similar action on biofilms, including those of *Staphylococcus aureus* [34, 38], the most common bacterial isolate in distal limb wounds [18, 66]. The supposed absence of bacterial resistance against Manuka has so far only been trialed on 60 common bacterial species (some known to be resistant to antibiotics), which were immobilised by medical-grade Manuka [19]. Needless to say, far more research into resistance against Manuka is required. Moreover, further research is needed to solidify current assumptions *in vivo* as most research has been trialed *in vitro*.

Treatment of Equine Distal Limb Wounds with Manuka Honey

In total, 4 noteworthy publications describing the affect of Manuka on the second-intention healing have been published. In the earliest of these [11], surgical EDLWs were created and contaminated with faeces for 24 hours (allowing for sufficient contamination [17]). The contamination with faeces mirrors the common gross contamination to which distal limbs are exposed [66]. The results were the first ever to show that the treatment of equine distal limb wounds with Manuka honey gel (MHG) had decreased wound retraction compared to untreated wounds, though healing time barely varied. This method was almost identically replicated in the 3 studies that followed, likely due to some authors being common to all papers, with slightly differing treatment selections. The 2012 study compared 5 treatments: 12 days of Manuka honey (MH), 12 days of MHG, 12 days of control gel, MHG throughout healing, and an untreated control [12]. It showed that wounds treated with the long term MHG healed notably faster than all other wounds throughout. These results contradicted the prior paper. In 2015, 3 non-Manuka treatment options were compared with MHG and an untreated control [13]. It found that there was no difference in healing time between treatments. Although the marginally shortest healing time belonged to Manuka treated wounds, it contradicts the 2013 results as this study found using MHG of no real benefit over notably cheaper and already established alternatives. The to date the most recent study [65], compared varying UMF levels with generic honey. Here it should be noted that all mentioned articles have authors in common, indicating the still rather small scope of this topic. The only clear evolution in method was the use of imaging software to measure wound area, improving accuracy. Results showed that the overall healing time of wounds treated with UMF20 honey was the fastest. This aligns with the results from the 2012 and 2015 studies, whilst rejecting those from 2011, suggesting a clear shift in the paradigm of this field and discrediting the early results. The results showed no notable difference in wound retraction between Manuka honey treated wounds and the controls, again contrasting the 2011 study. The similarity in method and authors would be expected to provide similar results, however here clear discrepancies are evident. Further evidence is irrefutably needed.

Scientific Research Question

How can the implementation of this procedure for the treatment of contaminated distal limb wounds using Manuka honey gel affect their healing time?

Objective

To predict the effect of the implementation of this procedure for the treatment of contaminated equine distal limb wounds using Manuka honey gel on their healing time.

Scientific Hypotheses

- **Null Hypothesis (H_0)**

It is expected that the implementation of this procedure for the treatment of contaminated EDLWs with Manuka Honey gel will have no effect on their healing time.

- **Hypothesis (H_1)**

It is expected that the implementation of this procedure for the treatment of contaminated EDLWs with Manuka Honey gel will reduce their healing time.

Associated Risks

Risks Associated with the Handling of Horses

Handling horses comes with significant risk, especially in scenarios of injury. It is of upmost importance that any immediate risks to people and/or horses are addressed prior to the commencement of the below proposed procedures and protocols. Only proceed when a safe environment has been established.

Risks related to Contamination

Wounds, and horses generally, are abundant sources of bacteria, including species that are linked to illness in humans and animals. Hence, apply correct hand washing and wound cleaning procedures to avoid contamination. Utilise only sterilised medical materials, to prevent contamination of the wound that may compromise the wound's healing and hence the results.

Proposed Protocols supporting Methodology

Assumptions made:

It is assumed that the procedures will only be performed by qualified veterinarians or veterinary nurses, who can refer back to their own acquired knowledge to assess and treat injuries via examination and have the tools to minimise risks accordingly. It is also assumed that participating veterinary practices have access to the equipment required to perform this procedure in an accurate, valid, and reliable manner.

Study Admission Criteria:

Equine is only to be admitted in to study if ALL criteria listed below are fulfilled. This is to control variables that may otherwise impact wound's healing.

Wound Markers

- Wound is located on the fore or hind distal limb and does not reach above hock/knee.
- Wound area does not exceed 30 cm^2 and can be measured using the ImageJ software, ie. Does not wrap around limb and/or can be entirely captured in photographs.
- Wound depth does not exceed normal full thickness wound depth (0.6 to 1 cm).
- Injury does not substantially interfere with vital organs, broken bones, tendons, joints or ligaments, such that these could alter healing times dramatically.
- Wound has not closed via primary closure and/or remodelling phase of healing has not commenced.
- Wound has not been exposed to any treatments or medicines. At most has been rinsed with water or bandaged.

Contamination Markers

- There are no identifiable objects or pieces of debris lodged within the wound.
- The wound has been exposed to some form of contamination, eg non-sterile bandages, has been left open for period of longer than 24 hours, wound occurred whilst out of human supervision.

Epidemiological Markers

- Owner of animal consents to participation in the trial.
- Equine age is within the permitted range of 5 to 20 years.
- Equine height is within range permitted: 14 to 18 h.h.
- Equine body condition[(1)] is within the healthy range of moderately thin to moderately fleshy.
- Equine is not a pony or draught breed.
- Equine free from significant chronic illness that may impact healing of wound (eg. cancer, laminitis, equine metabolic syndrome, etc.)

Elimination from Study:

The equine is to be eliminated from the study if ANY of the below occur.

- Equine obtains new injury that interferes with the wound being observed.
- Owner removes equine from trial or veterinary facility prior to healing completion.
- Measurements can no longer be taken for any reason or treatment is broken off.

Proposed Methodology and Apparatus

Upon admittance, steps to decrease immediate risks and establish a safe environment for the animals and humans present should be taken prior to commencement of the methodology below.

Data Collection and Treatment upon admission:

1. Initial wound data is collected. If ALL criteria outlined in *Wound Markers* are fulfilled progress to step (2), otherwise treat according to standard veterinary practice.
2. Wound area is recorded. A digital photograph is taken with a 1 cm x 1 cm template (1 cm^2) held next to the wound as a standard reference (repeat 3 times). Photos are analysed post treatment using image analysis software (ImageJ, US National Institutes of Health, Bethesda, MD, USA). The mean area (cm^2) is calculated from 3 images and recorded. If wound area does not exceed 30 cm^2 and is measurable using the ImageJ software, continue on to step (3), otherwise treat according to standard veterinary practices.
3. Contamination data is collected. If ALL criteria outlined in *Contamination Markers* are fulfilled continue on to step (4), otherwise treat according to standard veterinary practices.
4. Epidemiological data is collected. If ALL criteria outlined in *Epidemiological Markers* is fulfilled continue on to step (5), otherwise treat according to standard veterinary practices.
5. Wound is cleaned, treated and bandaged using Manuka Honey Gel (MHG) and sterile equipment via standard treatment and bandaging procedures.

Continuing Data Collection and Treatment:

1. Wound area to be measured weekly via aforementioned procedure and analysis. Mean area and day of healing are recorded.
2. Wound is cleaned, treated and bandaged daily using MHG and sterile equipment via standard treatment and bandaging procedures.

Continuing Data Collection and Treatment is to be repeated until healing is completed (granulation tissue is no longer visible) or until ANY of the criteria outlined in *Proposed Protocol supporting Methodology; Elimination from Study* are fulfilled.

Expected Results from Data Analysis

Prediction of Healing Times of Contaminated Distal Limb Wounds

Pre-existing data provided by Professor Andrew Dart from his 2012 study, *Effect of Short- And Long-Term Treatment With Manuka Honey on Second-Intention Healing of Contaminated Wounds on the Distal Aspect of the Forelimbs of Horses* [12], was reexamined using paired two-tail T-tests to predict the healing time of more realistic EDLWs.

This report proposes long term treatment (until healing completion) with Manuka Honey gel using an application method very similar to the one used in the 2012 study. Hence the data from this trial, and specifically from the wounds treated with the "Long-Term Manuka Honey + pH neutral, water-based gel" (LT MHG) treatment act as an effective model for the prediction of healing times/rates of real-life scenario wounds with the above proposed methodology and protocols. The study from which these results are sourced had wounds which were created to be full-thickness and 2 cm x 2 cm in area.

The first T-test (*Figure 3)*, comparing healing times of contaminated versus non-contaminated LT Mix treated wounds, shows that the contamination status of Long-Term Manuka honey gel treated wounds affects their healing rates (t-stat > t Critical two-tail and a P-value < 0.05) and that contaminated, LT MHG treated wounds were shown to heal at faster rates, in terms of wound contraction (mean = 0.117 cm^2/day), than their non-contaminated equivalents did (mean = 0.099 cm^2/day). This supports the notion that contamination can be combatted using long term application of MHG.

H0: No difference in healing rates of contaminated vs non-contaminated Long-Term Mix Treated

t-Test: Two-Sample Assuming Equal Variances

	Contaminated Long Term Mix	*Non-Contaminated Long Term Mix*
Mean	0,116666667	0,098888889
Variance	0,000225	0,000311111
Observations	9	9
Pooled Variance	0,000268056	
Hypothesized Mean Difference	0	
df	16	
t Stat	**2,303410402**	
P(T<=t) one-tail	0,017504964	
t Critical one-tail	1,745883676	
P(T<=t) two-tail	**0,035009928**	
t Critical two-tail	**2,119905299**	

Figure 3: A paired two-tail T-test showing that there is a difference in healing rates between contaminated and non-contaminated LT Mix treated EDLWs.

The contaminated wounds best model the realistic wound scenario which this report expects to treat as only surgically created wounds can be created truly free from contamination. Therefore only the data from contaminated wounds will be used to predict these expected results. It is hence predicted that realistic wounds treated utilising MHG via this procedure will heal at the same rate as LT Mix treated contaminated wounds.

The next T-test (*Figure 4*), comparing the healing time of untreated versus LT Mix treated contaminated EDLWs, shows that the there is a significant difference in healing time between contaminated LT Mix treated wounds and contaminated untreated wounds (t-stat > t Critical two-tail and a P-value < 0.05). It also showed that the LT Mix treated wounds healed, on average, 2 weeks faster (mean = 46.5 days) than contaminated untreated wounds (mean = 61.7 days).

From this data, the expected healing time of an untreated EDLWs can be extrapolated. Assuming that an average contaminated wound will have an area of approximately 20 cm^2, we can assume that this wound, if left untreated, would heal at a rate of 0.099 cm^2/day. This would mean that it would take around 202 days to heal. Comparatively, via this same approximation, a contaminated 20 cm^2 wound treated with Manuka Honey Gel (LT Mix) could be expected to heal at a rate of 0.117 cm^2/day, allowing it to heal within 171 days. The difference healing rate makes is evident in the difference of 4.5 weeks. This expected decrease in healing time is significant enough to support the implementation of the above proposed methodology and protocols.

H0: No difference in healing times of contaminated untreated vs Long-Term Mix Treated

t-Test: Two-Sample Assuming Equal Variances

	Contaminated Long Term Mix	*Contaminated Long Term Mix*
Mean	61,77777778	46,55555556
Variance	56,94444444	8,527777778
Observations	9	9
Pooled Variance	32,73611111	
Hypothesized Mean Difference	0	
df	16	
t Stat	**5,643789036**	
P(T<=t) one-tail	0,000018312	
t Critical one-tail	1,745883676	
P(T<=t) two-tail	**0,030000036**	
t Critical two-tail	**2,119905299**	

Figure 4: A paired two-tail T-test showing that there is a difference in healing times between contaminated untreated and LT Mix treated EDLWs.

Original data[2] and statistical analyses [3] are included in the appendices.

Discussion

The results showed that the implementation of the provided protocols and methodology are expected to lead to a dramatic decrease in the healing times of equine distal limb wounds. This was shown via extrapolation of the data which described the healing rates and times of a 2 cm x 2 cm wound treated with either Manuka Honey gel or left untreated. The data from this was used to create a linear model of healing from the healing rates of both the untreated (mean = 0.099 cm^2/day) and MHG treated wounds (mean = 0.117 cm^2/day). This, however, assumed that the healing rates of a 4 cm^2 and 20 cm^2 are equivalent. This means that the results inherently carry inaccuracies as a variety of factors that influence the rate, such as perimeter length or granulation tissue formation, are not accounted for. Nonetheless, as the difference in expected overall healing time of the 20 cm^2 wound

was so significant (approximately 4.5 weeks), this discrepancies aren't expected to affect the overall result which shows that MHG treated wounds would heal faster than untreated wounds. The healing time for the 20 cm^2 exemplar wound could have also been calculated from the average healing time of a 2 cm x 2 cm wound (with an area of 4 cm^2) which takes 61,7 days to heal. Extrapolating from this, a 20 cm^2 wound would take 308 days to heal. This measure, however, is less precise as wound repair is known to progress inwards from the perimeter and a 20 cm^2 wound would never have an 8 cm perimeter (like the 2 cm x 2 cm wound used as a model). Hence, 202 days is a better approximation of healing time. It should be noted that these numerical values are purely approximations and highly inaccurate. The results are based on reasonably reliably data, as both the MHG and control group consisted of 9 seperate wounds on different horses. Still, to either solidify or disprove these results the proposed methodology and protocol would actually have to be implemented in veterinary practices for an extended period of time and conducted as an experimental trial. This type of study is required anyway to support the very primitive data currently available justifying Manuka Honey gel's current application on EDLWs (as highlighted in *Literature Review; Treatment of Equine Distal Limb Wounds with Manuka Honey*). If the proposed study were to applied, a variety of variables would have to be considered. As honey quality consistently fluctuates, the variables in a long term study would be difficult to keep accurate and controlled, especially since the action of Manuka as a wound healing agent isn't fully explored nor explained yet. These results were based on only 18 wounds, which in no way are representative of realistic wounds. So if I were able to, the next phase of this project would include approaching veterinary clinics to implement my methodology and protocols. I would most likely have to specify variables even more definitively. This, predictably, would be quite difficult as to compare real-life in vivo wounds, a vast amount of factors have to be taken into consideration. Finally, as this methodology follows the procedures utilised in the aforementioned studies I could only assume that a different methodology may provide greatly different results. I relied on the methodologies used in those studies as that would mean that my expected results could come as close as possible to their real results as possible.

Conclusion

In conclusion symposium, the use of pre-existing data supports that the implementation of the proposed methodology and accompanying protocols to treat contaminated equine distal limb wounds using Manuka honey gel, is expected to significantly decrease the healing rates and times of these wounds. This supports the notion that medical-grade Manuka Honey gel is a viable option for the future of veterinary wound treatment.

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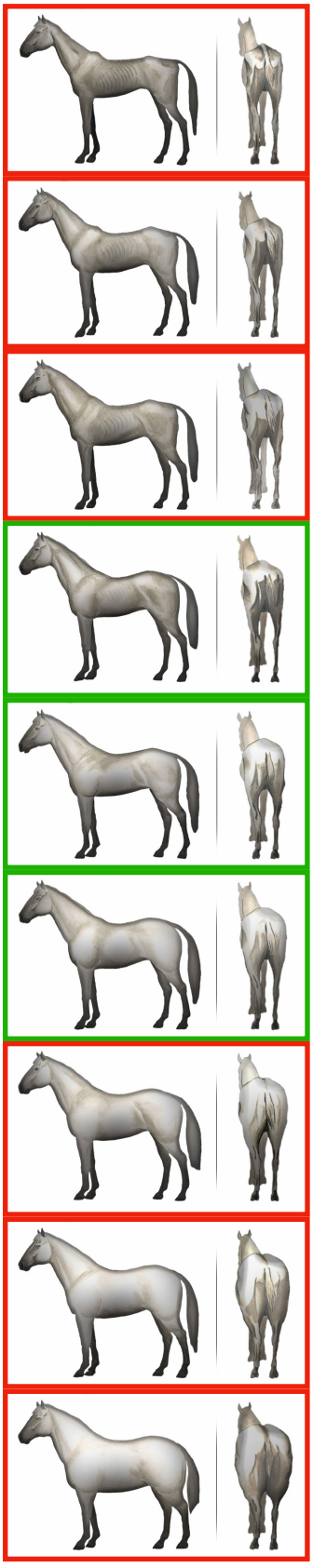
Appendices

[1] Body Condition Score Scale:



1. Extremely Thin
2. Very Thin
3. Thin
4. Moderately Thin
5. Moderate
6. Moderately Fleshy
7. Fleshy
8. Very Fleshy
9. Very Fleshy

Outside Healthy Range

Permitted Healthy Range

Outside Healthy Range

[2] Original Data provided by Prof. Andrew Dart:

- Key for colour coordination:

Key	
Control:	no treatment
Honey	pure manuka Honey
Mix - LT	Manuka honey + gel applied for a long term
Gel:	pH neutral, water based gel
Mix - ST	Manuka honey + gel applied for a short term

- Raw Data (sorted) from *Effect of Short- And Long-Term Treatment With Manuka Honey on Second-Intention Healing of Contaminated Wounds on the Distal Aspect of the Forelimbs of Horses* (2012)

Horse	Contamination Status	Wound	Treat	Healing time (days)	Healing Rate (cm2/day)				
38	contaminated	Left 1	Control	74	0,1		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	contaminated	Left 4	Control	62	0,08	MEAN	57,27272727		0,082
40	contaminated	Left 2	Control	59	0,08	STD DEV	8,096638534		0,013165612
41	contaminated	Left 3	Control	59	0,1				
42	contaminated	Left 4	Control	58	0,07	CORRELATION	-0,198045645		
43	contaminated	Right 2	Control	61	0,08	rank: 8	not correlated		
44	contaminated	Right 2	Control	57	0,07				
46	contaminated	Right 3	Control	72	0,07				
47	contaminated	Right 4	Control	76	0,07				
48	contaminated	Right 4	Control	52	0,1				
38	non-contaminated	Right 2	Control	62	0,09		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	non-contaminated	Right 4	Control	77	0,06	MEAN	59,81818182		0,077
40	non-contaminated	Right 5	Control	67	0,08	STD DEV	7,036413228		0,009486833
41	non-contaminated	Right 1	Control	66	0,07				
42	non-contaminated	Right 5	Control	58	0,07	CORRELATION	-0,009987025		
43	non-contaminated	Left 5	Control	61	0,08	rank: 10	not correlated		
44	non-contaminated	Left 1	Control	66	0,08				
46	non-contaminated	Left 5	Control	61	0,07				
47	non-contaminated	Left 3	Control	79	0,09				
48	non-contaminated	Left 4	Control	61	0,08				
38	contaminated	Left 4	Honey	57	0,08		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	contaminated	Left 1	Honey	52	0,13	MEAN	46,90909091		0,099
40	contaminated	Left 4	Honey	53	0,09	STD DEV	4,115013163		0,021832697
41	contaminated	Left 2	Honey	45	0,12				
42	contaminated	Left 2	Honey	52	0,08	CORRELATION	-0,623317624		
43	contaminated	Right 1	Honey	51	0,12	rank: 3	moderately correlated		
44	contaminated	Right 4	Honey	47	0,1				
46	contaminated	Right 2	Honey	58	0,06				
47	contaminated	Right 1	Honey	53	0,11				
48	contaminated	Right 5	Honey	48	0,1				
38	non-contaminated	Right 4	Honey	54	0,07		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	non-contaminated	Right 3	Honey	62	0,07	MEAN	43,45454545		0,092222222
40	non-contaminated	Right 1	Honey	55	0,11	STD DEV	6,293735863		0,017873009
41	non-contaminated	Right 5	Honey	45	0,11				
42	non-contaminated	Right 1	Honey	49	0,1	CORRELATION	-0,502522753		
44	non-contaminated	Left 3	Honey	47	0,11	rank: 6	moderately correlated		
46	non-contaminated	Left 4	Honey	56	0,07				
47	non-contaminated	Left 5	Honey	62	0,1				
48	non-contaminated	Left 2	Honey	48	0,09				
43	non-contaminated	Left 2	Honey	na	na				
				NOTE: ^inherant error, reliability compromised					
38	contaminated	Left 5	Mix- LT	49	0,15		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	contaminated	Left 5	Mix - LT	49	0,09	MEAN	42,54545455		0,12
40	contaminated	Left 1	Mix - LT	49	0,1	STD DEV	2,859681412		0,017638342
41	contaminated	Left 1	Mix- LT	42	0,14				
42	contaminated	Left 3	Mix - LT	42	0,13	CORRELATION	-0,396510399		
43	contaminated	Right 5	Mix - LT	46	0,11	rank: 7	not correlated		
44	contaminated	Right 1	Mix - LT	46	0,12				
46	contaminated	Right 1	Mix - LT	48	0,12				
47	contaminated	Right 5	Mix - LT	50	0,12				
48	contaminated	Right 1	Mix - LT	47	0,12				
38	non-contaminated	Right 5	Mix- LT	52	0,1		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	non-contaminated	Right 5	Mix - LT	51	0,08	MEAN	43,90909091		0,099
40	non-contaminated	Right 2	Mix - LT	49	0,09	STD DEV	4,715223572		0,0166333
41	non-contaminated	Right 2	Mix - LT	43	0,09				
42	non-contaminated	Right 4	Mix - LT	42	0,08	CORRELATION	-0,123252584		
43	non-contaminated	Left 1	Mix - LT	51	0,11	rank: 9	not correlated		
44	non-contaminated	Left 2	Mix - LT	47	0,1				
46	non-contaminated	Left 2	Mix - LT	50	0,12				
47	non-contaminated	Left 1	Mix - LT	56	0,09				
48	non-contaminated	Left 5	Mix - LT	42	0,13				

38	contaminated	Left 2	Gel	62	0,08		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	contaminated	Left 2	Gel	67	0,07	MEAN	56,81818182		0,081
40	contaminated	Left 3	Gel	63	0,08	STD DEV	7,397447007		0,01197219
41	contaminated	Left 5	Gel	59	0,07				
42	contaminated	Left 1	Gel	59	0,1	CORRELATION	-0,721389592		
43	contaminated	Right 3	Gel	72	0,07	rank: 1	correlated		
44	contaminated	Right 3	Gel	59	0,09				
46	contaminated	Right 4	Gel	51	0,1				
47	contaminated	Right 2	Gel	76	0,07				
48	contaminated	Right 3	Gel	57	0,08				
38	non-contaminated	Right 3	Gel	75	0,07		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	non-contaminated	Right 2	Gel	77	0,06	MEAN	59,45454545		0,078
40	non-contaminated	Right 4	Gel	67	0,06	STD DEV	11,59693446		0,022997584
41	non-contaminated	Right 4	Gel	52	0,07				
42	non-contaminated	Right 2	Gel	66	0,06	CORRELATION	-0,504934082		
43	non-contaminated	Left 4	Gel	61	0,06	rank: 5	moderately correlated		
44	non-contaminated	Left 5	Gel	63	0,09				
46	non-contaminated	Left 1	Gel	72	0,1				
47	non-contaminated	Left 4	Gel	79	0,08				
48	non-contaminated	Left 3	Gel	42	0,13				
38	contaminated	Left 3	Mix - ST	54	0,13		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	contaminated	Left 3	Mix - ST	52	0,08	MEAN	48,36363636		0,094
40	contaminated	Left 5	Mix - ST	54	0,11	STD DEV	4,211096453		0,022211108
41	contaminated	Left 4	Mix - ST	56	0,07				
42	contaminated	Left 5	Mix - ST	49	0,09	CORRELATION	-0,520314803		
43	contaminated	Right 4	Mix - ST	58	0,06	rank: 4	moderately correlated		
44	contaminated	Right 5	Mix - ST	58	0,08				
46	contaminated	Right 5	Mix - ST	56	0,1				
47	contaminated	Right 3	Mix - ST	45	0,12				
48	contaminated	Right 2	Mix - ST	50	0,1				
38	non-contaminated	Right 1	Mix - ST	60	0,08		HEALING TIME IN DAYS		RATE OF HEALING cm sq./day
39	non-contaminated	Right 1	Mix - ST	62	0,09	MEAN	49,54545455		0,09
40	non-contaminated	Right 3	Mix - ST	56	0,1	STD DEV	8,514693183		0,015634719
41	non-contaminated	Right 3	Mix - ST	45	0,11				
42	non-contaminated	Right 3	Mix - ST	45	0,08	CORRELATION	-0,692749935		
43	non-contaminated	Left 3	Mix - ST	62	0,08	rank: 2	correlated		
44	non-contaminated	Left 4	Mix - ST	48	0,09				
46	non-contaminated	Left 3	Mix - ST	63	0,07				
47	non-contaminated	Left 2	Mix - ST	62	0,08				

- Raw Data (sorted) from *A Preliminary Study on the Effect of Manuka Honey on Second-Intention Healing of Contaminated Wounds on the Distal Aspect of the Forelimbs of Horses* (2011)

horse	limb	treat	Day1 (W1)	Day7 (W2)	Day14 (W3)	Day21 (W4)	Day28 (W5)	Day35 (W6)	Day42 (W7)	Day56 (W8)	Day63 (W9)	Days to healing	Rate of Healing (cm2/day)
1	L	Control	8,52	15,73	15,14	14,27	8,91	6,57	5,11	4,08	3,94	127	0,067
2	R	Control	7,55	11,38	10,05	7,54	5,08	3,5	2,21	1,51	1,72	113	0,067
3	L	Control	6,07	10,96	10,46	6,13	3,67	2,33	1,63	1,13	1,13	113	0,054
5	L	Control	6,99	10,19	6,6	4,55	2,63	1,88	1,31	0,93	0,82	90	0,078
6	R	Control	7,19	11,89	11,3	9,41	6,68	4,17	3,5	2,72	2,49	128	0,056
7	L	Control	6,7	10,34	7,45	8,72	3,98	2,46	1,66	1,2	0,96	112	0,06
8	R	Control	6,41	11,71	12,38	11,12	6,55	5,12	3,9	3,06	2,32	94	0,068
9	L	Control	5,7	12,24	9,95	6,49	3,99	2,77	2,9	1,47	0,88	97	0,059
1	R	Honey	6,8	10,74	9,09	6,88	3,1	2,19	1,64	1,42	0,89	97	0,07
2	L	Honey	8,56	8,72	7,33	5,55	3,55	2,12	1,75	1,37	1,09	114	0,075
3	R	Honey	7,53	10,7	6,35	4,01	2,56	1,6	2,14	1,58	1,28	112	0,067
5	R	Honey	7,93	11,01	7,73	4,27	2,16	1,34	1,37	0,66	0,51	108	0,073
6	L	Honey	7,76	10,07	7,49	5,15	3,28	2,16	1,88	1,13	0,73	104	0,075
7	R	Honey	8,13	9,26	9,31	4,75	2,23	1,44	1,36	1,12	0,78	113	0,072
8	L	Honey	6,14	9,97	8,47	4,29	3,02	2,31	1,75	1,44	1,12	94	0,065
9	R	Honey	6,02	9,86	8,55	5,77	4,1	2,38	1,73	2,11	2,27	106	0,057

(3) All statistical tests performed:

- From 2012 study:

T-Test Rules:						
alpha = 0.05						
Reject H0 if:	**Assume H0 if:**					
P(T<=t) two-tail < 0.05	P(T<=t) two-tail > 0.05					
t-stat > t Critical two-tail	t-stat < t Critical two-tail					
H0: No difference in healing times of contaminated VS non-contaminated control treated wounds				**H0: No difference in healing rates of contaminated VS non-contaminated control treated wounds**		
t-Test: Two-Sample Assuming Equal Variances				t-Test: Two-Sample Assuming Equal Variances		
	Contaminated Control	*Non-Contaminated Control*			*Contaminated Control*	*Non-Contaminated Control*
Mean	61,77777778	66,22222222		Mean	0,08	0,075555556
Variance	56,94444444	53,69444444		Variance	0,00015	7,77778E-05
Observations	9	9		Observations	9	9
Pooled Variance	55,31944444			Pooled Variance	0,000113889	
Hypothesized Mean Difference	0			Hypothesized Mean Difference	0	
df	16			df	16	
t Stat	-1,267607598			t Stat	0,883452209	
P(T<=t) one-tail	0,11153811			P(T<=t) one-tail	0,195036844	
t Critical one-tail	1,745883676			t Critical one-tail	1,745883676	
P(T<=t) two-tail	0,22307622			P(T<=t) two-tail	0,390073689	
t Critical two-tail	2,119905299			t Critical two-tail	2,119905299	
t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05				t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05		
therefore we assume H0				therefore we assume H0		
therefore contamination status has no effect on healing times of control treated wounds				therefore contamination status has no effect on healing rates of control treated wounds		
H0: No difference in healing times of contaminated Vs non-contaminated Honey treated wounds				**H0: No difference in healing rates of contaminated Vs non-contaminated Honey treated**		
t-Test: Two-Sample Assuming Equal Variances				t-Test: Two-Sample Assuming Equal Variances		
	Contaminated Honey	*Non-Contaminated Honey*			*Contaminated Honey*	*Non-Contaminated Honey*
Mean	51	53		Mean	0,101111111	0,095
Variance	15	45,14285714		Variance	0,000486111	0,000285714
Observations	9	8		Observations	9	8
Pooled Variance	29,06666667			Pooled Variance	0,000392593	
Hypothesized Mean Difference	0			Hypothesized Mean Difference	0	
df	15			df	15	
t Stat	-0,763438694			t Stat	0,634732765	
P(T<=t) one-tail	0,228521109			P(T<=t) one-tail	0,267582484	
t Critical one-tail	1,753050356			t Critical one-tail	1,753050356	
P(T<=t) two-tail	0,457042217			P(T<=t) two-tail	0,535164969	
t Critical two-tail	2,131449546			t Critical two-tail	2,131449546	
t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05				t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05		
therefore we assume H0				therefore we assume H0		
therefore contamination status has no effect on healing times of Honey treated wounds				therefore contamination status has no effect on healing rates of pure Manuka Honey treated wounds		
H0: No difference in healing times of contaminated vs non-contaminated Long-Term Mix Treated				**H0: No difference in healing rates of contaminated vs non-contaminated Long-Term Mix Treated**		
t-Test: Two-Sample Assuming Equal Variances				t-Test: Two-Sample Assuming Equal Variances		
	Contaminated Long Term Mix	*Non-Contaminated Long Term Mix*			*Contaminated Long Term Mix*	*Non-Contaminated Long Term Mix*
Mean	46,55555556	47,88888889		Mean	0,116666667	0,098888889
Variance	8,527777778	23,11111111		Variance	0,000225	0,000311111
Observations	9	9		Observations	9	9
Pooled Variance	15,81944444			Pooled Variance	0,000268056	
Hypothesized Mean Difference	0			Hypothesized Mean Difference	0	
df	16			df	16	
t Stat	-0,711130621			t Stat	2,303410402	
P(T<=t) one-tail	0,243620937			P(T<=t) one-tail	0,017504964	
t Critical one-tail	1,745883676			t Critical one-tail	1,745883676	
P(T<=t) two-tail	0,487241875			P(T<=t) two-tail	0,035009928	
t Critical two-tail	2,119905299			t Critical two-tail	2,119905299	
t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05				t Stat > t Critical two-tail AND P(T<=t) two-tail < 0.05		
therefore we assume H0				therefore we reject H0		
therefore, contamination status has no affect on heling times of LT Mix treated wounds				therefore, contamination status does affect healing rates of Long Term Mix treated wounds		

H0: There is no difference in healing times of contaminated Honey treated vs Control treated wounds		
t-Test: Two-Sample Assuming Equal Variances		
	Contaminated Control	*Contaminted Honey*
Mean	61,77777778	51
Variance	56,94444444	15
Observations	9	9
Pooled Variance	35,97222222	
Hypothesized Mean Difference	0	
df	16	
t Stat	3,811990836	
P(T<=t) one-tail	0,000766723	
t Critical one-tail	1,745883676	
P(T<=t) two-tail	0,001533447	
t Critical two-tail	2,119905299	
t Stat > t Critical two-tail AND P(T<=t) two-tail < 0.05		
therefore we reject H0		
therefore there IS a significant difference in healing times of cont. Honey vs Conrtol treated wounds		

	Contaminated Control	*Contaminated Long Term Mix*
Mean	61,77777778	46,55555556
Variance	56,94444444	8,527777778
Observations	9	9
Pooled Variance	32,73611111	
Hypothesized Mean Difference	0	
df	16	
t Stat	5,643789036	
P(T<=t) one-tail	1,8312E-05	
t Critical one-tail	1,745883676	
P(T<=t) two-tail	3,6624E-05	
t Critical two-tail	2,119905299	
t Stat > t Critical two-tail AND P(T<=t) two-tail < 0.05		
therefore we reject H0		
therefore there is a dif in healing times of contaminated Contro vs Mix Long term treated wounds		

H0: No difference in healing times of contaminated vs non-contaminated Hydrogel Treated

t-Test: Two-Sample Assuming Equal Variances

	Contaminated Hydrogel	*Non-Contaminated Hydrogel*
Mean	62,55555556	64,33333333
Variance	61,52777778	138,5
Observations	9	9
Pooled Variance	100,0138889	
Hypothesized Mean Difference	0	
df	16	
t Stat	-0,37709743	
P(T<=t) one-tail	0,355527977	
t Critical one-tail	1,745883676	
P(T<=t) two-tail	0,711055954	
t Critical two-tail	2,119905299	

t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05
therefore we assume H0
therefore, contamination status has no affect on healing times of hydrogel treated wounds

H0: No difference in healing rates of contaminated vs non-contaminated Hydrogel Treated

t-Test: Two-Sample Assuming Equal Variances

	Contaminated Hydrogel	*Non-Contaminated Hydrogel*
Mean	0,081111111	0,078888889
Variance	0,000161111	0,000586111
Observations	9	9
Pooled Variance	0,000373611	
Hypothesized Mean Difference	0	
df	16	
t Stat	0,243884304	
P(T<=t) one-tail	0,405210032	
t Critical one-tail	1,745883676	
P(T<=t) two-tail	0,810420065	
t Critical two-tail	2,119905299	

t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05
therefore we assume H0
therefore, contamination status has no affect on healing rates of hydrogel treated wounds

H0: No difference in healing times of contaminated vs non-contaminated Short-Term Mix Treated

t-Test: Two-Sample Assuming Equal Variances

	Contaminated Short Term Mix	*Non-Contaminated Short Term Mix*
Mean	53,11111111	55,375
Variance	19,86111111	65,69642857
Observations	9	8
Pooled Variance	41,25092593	
Hypothesized Mean Difference	0	
df	15	
t Stat	-0,725404165	
P(T<=t) one-tail	0,23968466	
t Critical one-tail	1,753050356	
P(T<=t) two-tail	0,479369321	
t Critical two-tail	2,131449546	

t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05
therefore we assume H0
therefore, contamination status has no affect on healing times of ST Mix treated wounds

H0: No difference in healing rates of contaminated vs non-contaminated Short-Term Mix Treated

t-Test: Two-Sample Assuming Equal Variances

	Contaminated Short Term Mix	*Non-Contaminated Short Term Mix*
Mean	0,09	0,0875
Variance	0,000375	0,000164286
Observations	9	8
Pooled Variance	0,000276667	
Hypothesized Mean Difference	0	
df	15	
t Stat	0,309316707	
P(T<=t) one-tail	0,380667856	
t Critical one-tail	1,753050356	
P(T<=t) two-tail	0,761335712	
t Critical two-tail	2,131449546	

t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05
therefore we assume H0
therefore, contamination status has no affect on healing rates of ST Mix treated wounds

- From 2011 study:

H0: That there is a difference between healing times of manuka and control treated contaminated wounds

t-Test: Two-Sample Assuming Equal Variances

	Control	*Manuka Honey*
Mean	106,7142857	107,2857143
Variance	182,5714286	48,23809524
Observations	7	7
Pooled Variance	115,4047619	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0,099513983	
P(T<=t) one-tail	0,461186664	
t Critical one-tail	1,782287556	
P(T<=t) two-tail	0,922373329	
t Critical two-tail	2,17881283	

t Stat < t Critical two-tail AND P(T<=t) two-tail >0.05
therefore we assume null hypothesis

H0: There is no dif in wound area between honey and control treated wounds

DAY 1

t-Test: Two-Sample Assuming Equal Variances

	Control	*Honey*
Mean	6,658571429	7,43857143
Variance	0,419280952	0,96458095
Observations	7	7
Pooled Variance	0,691930952	
Hypothesized Mean Differenc	0	
df	12	
t Stat	-1,754273268	
P(T<=t) one-tail	0,052429949	
t Critical one-tail	1,782287556	
P(T<=t) two-tail	0,104859898	
t Critical two-tail	2,17881283	

t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05
therefore we assume H0

DAY 7

t-Test: Two-Sample Assuming Equal Variances

	Control	*Honey*
Mean	11,24428571	9,941428571
Variance	0,608961905	0,616247619
Observations	7	7
Pooled Variance	0,612604762	
Hypothesized Mean Differe	0	
df	12	
t Stat	3,114157951	
P(T<=t) one-tail	0,004475717	
t Critical one-tail	1,782287556	
P(T<=t) two-tail	0,008951433	
t Critical two-tail	2,17881283	

t Stat > t Critical two-tail AND P(T<=t) two-tail < 0.05
therefore we reject H0

DAY 14

t-Test: Two-Sample Assuming Equal Variances

	Control	*Honey*
Mean	9,74142857	7,89
Variance	4,19424762	0,9432
Observations	7	7
Pooled Variance	2,56872381	
Hypothesized Mean Differen	0	
df	12	
t Stat	2,1611369	
P(T<=t) one-tail	0,02580316	
t Critical one-tail	1,78228756	
P(T<=t) two-tail	0,05160633	
t Critical two-tail	2,17881283	

t stat < t Critical two-tail AND P(T<=t) two-tail > 0.05
therefore we assume H0