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Investigating the Antibacterial Properties of Leech Saliva: Starvation and Dose-Dependent Effect of Crude Leech Saliva Extraction (CLSE) on Bacterial Strains

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ABSTRACT

The Asian buffalo leech, *Hirudinaria manillensis*, emerges as a prominent species utilized for medicinal purposes due to its physiological similarities to the European medicinal leech. Hirudotherapy is renowned for its diverse mechanisms of action, including the anticoagulant properties of hirudin found in leech saliva, which inhibit thrombin and prevent blood clotting. This study investigates the impact of feeding 60 leeches on their weight gain and protein concentration in their saliva over an 8-week starvation period, and the antibacterial efficacy of crude leech saliva extraction (CLSE) against *E. coli*, *Klebsiella* sp., and *Salmonella* sp. To the starved leech, the weight gain was highest in the 2nd week (55.06%), 38.82% in the 4th week and followed by a 50.68% increase in the 8th week. The protein concentrations, measured using the Bradford assay, were highest in the 4th week starvation and declined thereafter. The CLSE concentrations tested at 25%, 50%, 75%, and 100% showed increased inhibition zones for all bacterial strains, with *Klebsiella sp*. demonstrating the highest sensitivity on the 4th week starved leeches. On the 4th week starvation, the *Salmonella sp.* strains exhibited inhibition zones ranging from 15 mm to 35 mm, while *Klebsiella sp*. strains ranged from 25 mm to 45 mm. *E. coli* strains showed a less pronounced response. The positive control (Chloramphenicol) showed a 35 mm inhibition zone. These findings indicate that CLSE has a starvation time and dose-dependent antibacterial effect, particularly against *Klebsiella sp*. Further research is recommended to understand the factors affecting protein concentration in leech saliva and to optimize CLSE's antibacterial properties.

Keywords: *Hirudinaria manillensis,* Antibacterial activity, Crude leech saliva extraction, Starvation, Protein concentration.

1. INTRODUCTION

In several scientific disciplines, including toxicology, physiology, neurology, biochemistry, and histology, leeches are a common choice for animal models. Leeches have been used for therapeutic and medical purposes since the fifth century BC. Following that, it saw significant use in the 19th century before declining in the early 20th century (Papavramidou & Christopoulou-Aletra, 2009). In Southeast Asia, natural water bodies are a popular habitat for the Asian buffalo leech, or *Hirudinaria manillensis*, also referred to as the Asian medical leech. When fully extended, adults can stretch up to 200 mm, with a maximum width of 13 mm and length of 130–140 mm. They live in slow-moving or stagnant waterways, usually in rice paddies, swamps, and languid streams, throughout the Indo-West Pacific region. They are commonly

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discovered attached to livestock's stomachs, sucking on their blood, throughout the region's cattle-rearing nations (Kutschera & Roth, n.d). In the wild, they have been observed to consume mammals, reptiles, amphibians, and, much less frequently, Telostei fish. The Asian buffalo leech has no recognized predators as of yet. Due to its similar physiology and the European medicinal leech *Hirudo medicinalis* current conservation status, *H. manillensis* has become the more popular medicinal leech (Liu et al., 2023).

The use of leeches in medical treatments has a long history dating back over 2500 years, originating in ancient Egypt where they were employed for bloodletting. In literature, the leech therapy was dated and recorded in ancient Ayurveda Indian text, "Jalaukavacharan" which had been used there for blood purification. French doctor Francois Broussais in 19th century popularized leech therapy for treatment for range of ailments but there were concerns grown about disease transmission resulted in decline of the trend, while pathology, physiology, and microbiology had questioned their ailments. Leech therapy in mid-20th century again had resurgence to address treatments in microsurgeries to address issues pertaining to venous congestion after tissue reattachments, known as hirudotherapy for enduring the highlights for leech (Itrat & Haque, 2013).

Hirudotherapy, commonly known as leech therapy, is renowned for its diverse mechanisms of action. The majority of therapy adds to its effectiveness in healing. Many reports have shown that the bioactive compound Hirudin. This compound has strong anti-coagulant properties that block thrombin. Apart from hirudin, other inhibitors such as bufrudin and haemadin, broaden the range of anticoagulant activity across different leech species. A wide range of bioactive chemicals that can both reduce pain and prevent blood clotting are found in Leech species. In Leeches analgesic benefits have been noted for several ailments, such as osteoarthritis, iliasacral joint pain, and cervicobrachialgia syndrome. Hirudin can reduce synovial inflammation in patients with arthritis (Shakouri & Wollina, 2021).

Additionally, leech therapy is used in reconstructive surgery, where its capacity to lessen venous congestion and blood coagulation. These mechanisms aids in the process of revascularization after organ transplantation. In leech resection and replantation, such as penile replantation, emphasizes its effectiveness in promoting normal blood flow and favourable tissue outcomes (Guan et al., 2020). Leeches have been recognized as a promising treatment in reconstructive surgery. Some species show Y-shaped wounds resulting from their bites typically heal without scarring or complications. Leeches are useful for treating localized venous congestion and blood coagulation. This helps enhance blood flow and revascularization after organ transplant procedures, including those involving the fingers, ears, and eyelids (Sig, Guney, Uskudar Guclu, & Ozmen, 2017). Similarly, bloodletting with leeches, combined with vascular endothelial growth factor (VEGF), may enhance flap survival. There are many cases of leech resection and replantation, including a woman who had basal cell carcinoma over her nose. After 9 months of leech therapy, the women had normal blood circulation and a healthy flap (Anurag Srivastava & Ridhima Sharma, 2010). Additionally, penile replantation procedures without microsurgery achieved notable success when accompanied by hirudotherapy (Porshinsky, Saha, Grossman, Beery, & Stawicki, 2011).

In the realm of dental problems, several reports have mentioned the application of leeches in treating sublingual hematoma and preventing the formation of new blisters in patients with pemphigus, a skin disease with oral symptoms. Other studies have explored the use of leeches in treating gum diseases, where the direct application of 3 to 4 leeches at intervals of several days has proven to be a successful remedy for abscesses and inflammation. Leech therapy has also been notably effective in managing cases of macroglossia (lingual swelling), whether resulting from traumatic swelling due to facial injuries or non-traumatic causes like spinal surgery or cleft palate repair. The alleviation of macroglossia through leeching has been associated with a significant reduction in airway obstruction, leaving only minor post-treatment glossal puncture marks (Porshinsky et al., 2011).

In audiology and cases of ear abnormalities, therapists have utilized just two leeches, with one applied behind the ear and the other over the jaw in front of the ear. This treatment is repeated 2-3 times at intervals of 3-4 days, showing promising results for conditions such as tinnitus, despite the exact mechanism remaining unclear. Traditional therapists have also employed leeches for the healing of various skin disorders, despite the lack of scientific studies supporting this usage. For example, the usage of leeches for treating shingles, a viral skin infection (Facchin, Lancerotto, Arnež, Bassetto, & Vindigni, 2018). In terms of analgesic management, the pain-relieving effects of leech application have been confirmed in numerous trials on patients with osteoarthritis, who reported greater relief compared to topical Diclofenac, with no adverse effects. Animal studies have shown that hirudin can reduce synovial inflammation in arthritis patients. Furthermore, leech therapy has been indicated as an analgesic for conditions such as iliasacral joint pain and cervicobrachialgia syndrome (Rai, Singh, Dwivedi, Singh, & Rai, 2011). The leeches are fascinating creatures with medicinal properties. It was mentioned earlier that how long they go without food (starvation), how often we take their saliva (collections), and how well they bounce back after (recovery) all affect the amount of protein in their CLSE. This protein concentration turned out to be a significant factor in determining how well leech saliva works as medicine.

Despite this, there is no report available on how these CLSE proteins of leeches when they're starving fight against bacteria. Or if these starvation-induced proteins have any additional contribution to antimicrobial activity. Our investigation employed crude leech saliva extraction (CLSE) derived from *Hirudo manillensis* to explore the relationship between starvation duration and the resulting protein concentration alongside their corresponding microbial activity.

The objective of the study was to investigate the effectiveness of starvation and protein concentration levels on microbial activity using CLSE optimization in different dose variations

2. MATERIALS AND METHODS

2.1 Leech Feeding and Starvation

The leech was given a mixed solution of 0.001M Arginine (R&M Chemicals, United Kingdom) and 0.15M normal saline (Ain Medicare Sdn Bhd, Malaysia), which was heated to 37°C using a water bath, to encourage saliva collection after fasting for several months. The solution was then poured into the glass funnel and sealed with a piece of parafilm. The leech was weighed to determine its weight before it was positioned in front of the glass funnel on the parafilm. sheet. Leeches were weighed both before and after they sucked on the phagostimulatory fluid. Based on every trial, the average weight of the leech was determined. We attempted to gather saliva from unstimulated leeches but were unable to do so due to reports that leeches need to be starved for a specific amount of time to produce saliva. Therefore, a specific feeding schedule was implemented until we could gather enough and useful amounts of saliva extract for 2, 4 and 8 weeks. Leech acceptability of sucking the Phs was used to determine the minimum starvation period (Mohamed, Campbell, Cooper-White, Dimeski, & Punyadeera, 2012; Mohammed Abdualkader et al., 2013a). Before starting the extraction process, feeding operations are completed and the weight of each leech utilized is determined. The inverted funnel was fastened to the parafilm with the solution inside. The leech was positioned in the direction of the parafilm sheet, sucking the solution until it was satisfied and detached from its attachment on the sheet. The leeches were weighed again.

2.2 Leech Saliva Extract (CLSE)

The leeches were then placed inside the plastic bag and kept there for ten to fifteen minutes. After that, the leeches were paralyzed and placed on an ice pack. Massages were applied gradually from the rear to the front of the paralyzed leech. The leech's oral secretions were gathered and accumulated in vials. The liquid that had been collected was then centrifuged for 10 minutes at 4°C and 9000 rpm (Hettich). The supernatant that was left over after centrifugation was called crude leech saliva extract (CLSE) and was filtered through 0.45µm Sartorius filter paper. In this study, only fresh CLSE was employed. Blood mixed with crude saliva extraction was thrown away (Malik, Astuti, Arief, & Rahminiwati, 2019).

2.3 Body Weight

Starved leeches were fed on the Phagostimulatory solution through parafilm membrane sheets. Leech body weights before and after were recorded and the difference (body weight gain) was considered to resemble the volume of the sucked solution (A M Abdualkader, Ghawi, Alaama, Awang, & Merzouk, 2013; Abdualrahman Mohammed Abdualkader, Ghawi, Alaama, Awang, & Merzouk, 2013).

2.4 Total protein determination

The Bradford assay method was used to assess protein concentration. Distilled water was used as a blank on a cuvette at a wavelength of 595 nm, and bovine serum albumin (BSA) (R & M Chemicals, United Kingdom) was used as a standard in the calibration curve. Before usage, the spectrophotometer warmed up for 15 minutes. Meanwhile, 100 µl volumes of standards containing 25–150 µg BSA were generated. A volume of 1 ml dye reagent (R & M Chemicals, United Kingdom) was added, and the samples were incubated for 15 minutes. For one hour, the created compound remains stable. The same process was used to prepare the samples. As a result, the absorbance was measured at 595 nm, and a standard curve was created by plotting the absorbance against BSA content. The standard curve was used to determine the protein concentration, and the calibration curve equation was used to determine the saliva concentration (Lemke, Müller, Lipke, Uhl, & Hildebrandt, 2013; Shakouri et al., 2018).

A = m.C + constant

Whereas A = Absorbance at 595nm

C = Saliva protein concentration

m and constant = Constants from equation

2.5 Minimum inhibitory concentration test

The least concentration of fresh saliva extract that may stop the growth of isolates of bacteria like *Salmonella enterica* (ATCC13311), *Klebsiella pneumoniae* (ATCC13883), and *Escherichia coli* (ATCC10536) was determined using the minimum inhibitory concentration test. The first tube was filled with 10 ml of fresh leech saliva at a 100% saliva concentration to prepare serial dilutions. Then, with 7.5 milliliters of fresh saliva from the first tube and 2.5 milliliters of distilled water—a 75% solution concentration—in the second tube, the saliva was transferred. Thus, to create a 50% concentration solution, fresh saliva up to 6.67 ml was transferred from the second tube into the third tube, which contained 3.33 ml of distilled water. To create a 25% solution, 5 ml of fresh saliva solution from the third tube was taken and put into the last tube, which also included 5 milliliters of distilled water (HmbG Water Still 4L). Subsequently, the blank disc was put onto a 96-well microtitre plate that had labels reading 100%, 75%, 50%, and 25%, indicating varying concentrations. Up to 20µL of each solution from tube one through tube four was added to the first well to well four in each of the three rows that held the previously put blank disc. 10 minutes were spent incubating this procedure. Following incubation, Mueller Hinton Agar (MHA) (Oxoid Ltd, United Kingdom) streaked with the three tested species of bacteria was used to put the blank discs. The MHA will spend the next 18 to 24 hours in the incubator. When comparing the fresh saliva solution sample to Chloramphenicol (Oxoid Ltd, United Kingdom), which served as a positive control, and the blank disk as a negative control, the observation of MHA was observed (Kowalska-Krochmal & Dudek-Wicher, 2021). A 500 mg tablets of the antibiotic Ciprofloxacin (Ciproglen, India) were broken into powder. Next, the powdered antibiotic was weighed by the predetermined concentration order. Each Falcon tube is filled with a mixture of powder and one milliliter of distilled water to obtain a working concentration of 500 mg/mL and each well was loaded and adjusted for 5 mg in well or disc using the working stock of Chloramphenicol for 5 mg disk diffusion. The mixture was then centrifuged until it was dissolved. A syringe filter is used to filter the mixture once it has dissolved (Litwinowicz & Blaszkowska, 2014).

3. RESULTS

3.1 Leech Identification and feeding behavior

According to the taxonomy developed by Professors Roy T. Sawyer and Fred O.P. Hechtel, Leeches Biopharm (UK) Ltd., the medicinal Malaysian leeches employed in this study were *H. manillensis.* The solution was fed for one week following the starvation period. After feeding the solution, less than 30 of the 60 starving leeches agreed to suck the phagostimulary solution. Conversely, in these studies, leeches that were mostly hungry declined to drink the solution. During these studies, a lot of leeches attempted to suck the solution, and it took between 30 to 60 minutes to obtain the necessary saliva supply.

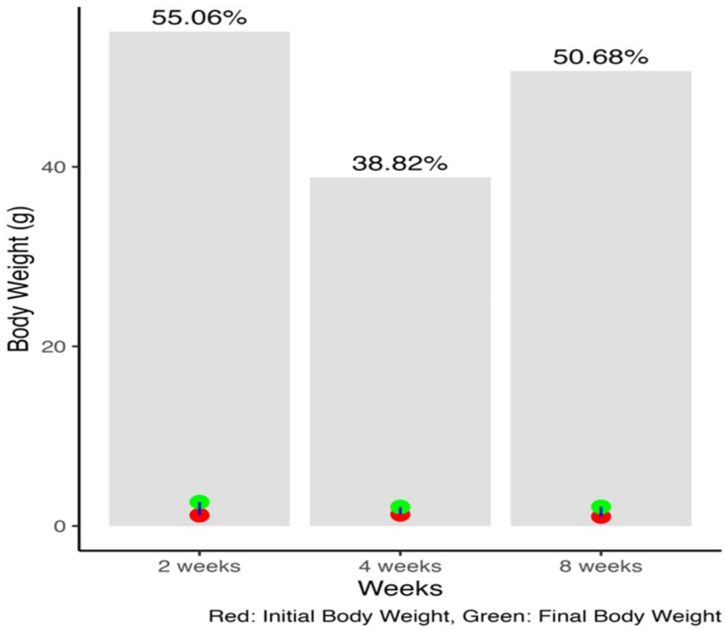
3.2 Body Weight Gaining

Feeding 60 leeches with an average body weight for each group for the time period specified in Table 1 in these studies demonstrates a substantial change in weight gain. A substantial weight rise of 55.06% occurred in the 2nd week of feeding, which was followed by a decrease in weight gain of 38.82% in the 4th week and a sharp increase in body weight of 50.68% in the 8th week, which was marginally lower than at the start of the trial. Nevertheless, throughout the trial, no statistically significant variations in weight increase were noted for any week (Table 1; Fig. 1).

Table 1: Body Weight Gain by the Starved Leeches in Weeks after Feeding (Mean ± SD)

Weeks	Initial body Weight (g)	Final Body Weight (g)	Body Weight Gain (%)
2 weeks	1.198 ± 0.436	2.666 ± 2.984	55.06
4 weeks	1.297 ± 0.517	2.120 ± 2.208	38.82
8 weeks	1.053 ± 0.566	2.135 ± 2.649	50.68

(A)



(B)

Fig. 1 (A): *Hirudinaria manillensis.* (B): Graphical representation of body weight gaining of leeches. The red dot inside the graph represents the weight of the starvation induced leeches and the green represents the body weight after feeding. The values are tabulated in Table 1.

3.3 Impact of Starvation on Leech Saliva Protein Concentration and Volume

Our results showed that starvation duration impacted the protein concentration in leech saliva. After depriving leeches of food for varying times, we observed a slight increase in protein concentration of 0.79% after 4 weeks of starvation, compared to 2 weeks. This peak reached 30.234 milligrams per milliliter (mg/mL) and coincided with a larger saliva volume of 18 microliters (µL). Leeches starved for 2 weeks produced saliva with a protein level of 29.998 mg/mL (12 µL volume), while those not fed for 8 weeks exhibited the lowest protein concentration of 26.310 mg/mL (with a mere 7 µL volume). Interestingly, we observed a decrease of 12.98% in protein concentration between 4 and 8 weeks of starvation (Table 2; Fig. 2). These findings suggest leeches can still produce protein even after extended fasting periods, but the total amount and volume of saliva produced significantly decrease with prolonged starvation.

Table 2: Saliva protein concentration and volume of CLSE

Group No.	Starvation Period	Total Protein Concentration (mg/ml ± SD)	Total Volume
1	2 weeks	29.998 ± 13.491	12 µL
2	4 weeks	30.234 ± 14.079	18 µL
3	8 weeks	26.310 ± 14.126	7 µL

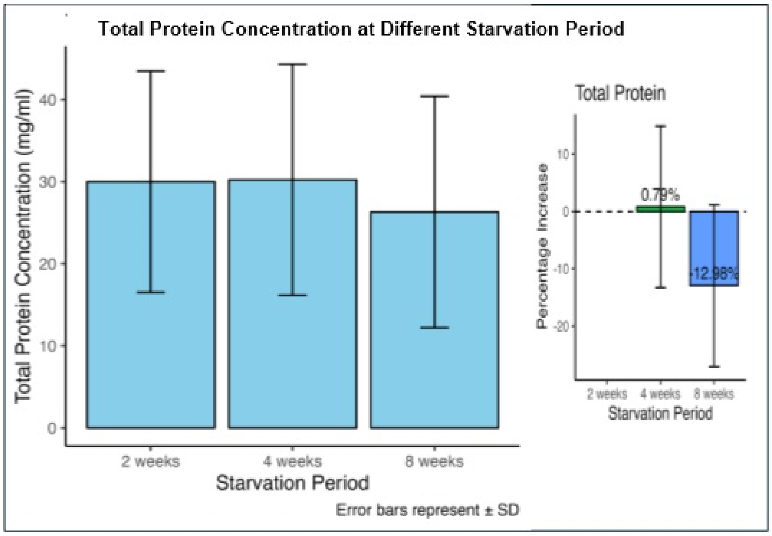


Fig 2: Total protein estimation on different starvation periods and the percentage variations.

The findings show that saliva with its protein content is secreted spontaneously whenever leeches sucke the fluid. The average volume of saliva that was successfully collected at the start of the second week was 14 uL, but the average volume increased somewhat the next week to 16.5 uL. However, the volume that was successfully collected at the conclusion of the eighth week was less than 5uL. The volume was calculated by averaging the number of leeches in each group that were able to successfully absorb the solution over the course of the hunger period.

3.4 Minimum Inhibition Concentration of Starvation period CLSE on the bacteria isolates

The CLSE from the 2nd, 4th and 8th week starvation was used 25%, 50%, 75% and 100% against 3 bacterial strains for their MIC to understand the changes of starvation period impacts the antibacterial activity (Table 3).

Table 3: MIC for the bacterial isolate for 2nd, 4th and 8th weeks.

Inoculum	Concentration (%) & Zone Inhibition (mm)				Positive Control	Negative Control
	25%	50%	75%	100%		
Salmonella sp. (2nd)	15	20	25	35	30mm	0
Salmonella sp.(4th)	20	25	30	35	30mm	0
Salmonella sp.(8th)	15	15	25	30	30mm	0
Klebsiella sp. (2nd)	25	30	30	45	35mm	0
Klebsiella sp. (4th)	25	35	40	45	35mm	0
Klebsiella sp. (8th)	25	30	30	40	35mm	0
E. coli (2nd)	20	25	35	35	30mm	0
E. coli (4th)	20	30	30	35	35mm	0
E. coli (8th)	20	25	25	30	35mm	0

The antibacterial properties of crude leech saliva extract (CLSE) obtained from *Hirudo manillensis* leeches subjected to varying starvation durations. The protein concentration of the CLSE was also analyzed. Ciprofloxacin served as the positive control for antibacterial activity. It was found that the optimal starvation period for antibacterial activity depended on the specific bacterial strain. Against *Salmonella* sp. and *Klebsiella* sp., CLSE from leeches starved for 4 weeks exhibited the most potent effect. This was characterized by inhibition zones reaching 35 and 45 mm, respectively at the highest concentration (100%), matching above the positive control (Ciprofloxacin). In contrast, *E. coli* displayed the greatest susceptibility to CLSE derived from leeches starved for 2 weeks. In this case, inhibition zones reached 30-35 mm at the higher concentrations (75% and 100%), equalling the positive control.

Overall, a clear trend emerged – higher concentrations of CLSE resulted in stronger antibacterial activity. For all bacterial strains tested (*Salmonella sp.*, *Klebsiella sp.*, and *E. coli)*, the 100% concentration of CLSE consistently produced the largest zones of inhibition. These findings suggest a dose-dependent and also effective at 4th week starvation period.

4. DISCUSSION

Based on their anatomical and morphological structures, the medicinal Malaysian leeches were identified as *H. manillensis*. Using 0.001M arginine in regular saline, phagostimulatory feeding was used to feed the medicinal varieties of Malaysian leeches. Parafilm membrane sheets and a glass funnel were among the feeding apparatuses employed. Surprisingly, there was insufficient antibacterial prophylaxis to guarantee total disinfection. Consequently, further microbial prophylaxis is required to eradicate any lingering leech in the digestive tract, making it more appropriate for patient therapy as it helps stop these germs from causing infection in the patient. Ciprofloxacin has consistently shown superior microbiological susceptibility and good preventive effectiveness against the majority of these organisms, making it an appropriate treatment for Aeromonas species linked to leech infection. Considering that Aeromonas species live in the intestinal tract of therapeutic leeches, their symbiotic association is not surprising (Lamy, Baron, & Barraud, 2021).

It was discovered that the Malaysian medical leech, *Hirudinaria manillensis*, must go through at least 21 days of hunger in the lab before being fed and having leech saliva extracted. Despite the paucity of research on Malaysian leeches, some investigators studying the therapeutic kind employed a two-week starving phase, which is very similar to our results (Alaama et al., 2024). In contrast to the European leech, Hirudinaria medicinalis has been the focus of numerous in-depth investigations worldwide. The duration of starvation varied greatly, ranging from four to six months. It is well known that certain leech species can retain blood meal in their bodies for up to 18 months (Baskova et al., 2008). In our results, there was weight gain after feeding between 55.06 to 38.82 % from their initial weight before feeding. During the feeding trials, there was a significant increase in body weight observed in the second week. However, by the fourth week, there was a reduction in weight gain. By the eighth week, the body weight increased sharply again, although this increase was slightly less than the initial weight at the beginning of the trial.

The study showed notable fluctuations in body weight, particularly in the 2nd and 8th weeks, where substantial increases were observed. In the 2nd week, there was an increase in the weight. This might indicate a period of rapid growth or an effective feeding regimen. The decline in the 4th week could suggest a temporary adaptation phase or a possible plateau in growth. The subsequent rise in the 8th week might imply a delayed growth spurt or a response to changes in feeding or environmental conditions. However, it is crucial to highlight that these variations were not statistically significant. Despite observable changes, the differences in weight gain could be due to random variation rather than consistent effects of the feeding trial. Therefore, any conclusions drawn from these fluctuations should be made cautiously, considering the lack of statistical significance.

Leeches can therefore remain satisfied for around 3 months after becoming full and having a digesting process. *H. manillensis* can be fed every 10 days, according to other reports. A solution with 150 mM NaCl and 1 mM arginine is equivalent to blood and can trigger the full feeding response as well as the consumption of *H. medicinalis*. It is believed that several processes had a role in the weight gain that was seen in *H. manilensis*. The following changes in their consumption of a phagostimulatory solution that contained arginine and regular saline. The development and protein synthesis of amino acids produce physiological functions that make arginine. Leeches may gain weight with an arginine-rich solution because it may promote enhanced protein synthesis and cell proliferation (Liu et al., 2023). Additionally, about half of the used starved leeches remained attached to the parafilm membrane for 20-40 mins during the feeding phase, exhibiting remarkable peristaltic contractions of the pharynx muscles that gradually slowed down and became less noticeable in the final stages of the feeding cycle before the leech stopped sucking and fell off the membrane (Mohammed Abdualkader et al., 2013a). In our study also the leech automatically fell off between 30 to 60 minutes.

The combining high-throughput proteomic and transcriptomic analyses is effective for determining wide profiles of proteins that are present at low concentrations in leech salivary secretions. This provides a new foundation for performing novel investigations and identifying potential pharmacological agents or targets in leech medicinal therapy. As a result, the variation in total protein concentration of crude leech saliva extraction from *H. manillensis* may not show significance in a Bradford assay (Mohammed Abdualkader et al., 2013b; Zheng, Li, Chen, Liu, & Chen, 2015). This was consistent with our findings that there was very little difference in the CLSE proteins at 2nd and 4th week concentrations—only a 0.79% increased. The properties of leech salivary secretions must be identified to employ these secretions medicinally. Thus, the molecular information provided by transcriptome and proteome studies can be used to get a deeper comprehension of the leech-sucking mechanism and its possible medical implications. It provided a novel approach for doing original research and locating putative pharmacological compounds or targets for therapeutic leech use (Liu et al., 2023). The effect of the starvation period on the protein content in the CLSE has a considerable effect on the quantity of proteins found in saliva from *H. manillensis*. Though the body composition was identical in all treatments, hungry leeches had the lowest quantities of crude fat and protein. This meant that fat and protein were broken down and used as fuel during the long sixty-day fast (Harvald et al., 2017).

The protein concentration decreased linearly over the eight weeks of the hunger strike; it began at 29.998±13.491 mg/ml in the first two weeks. There had been an approximate 78% increase by the fourth week of famine and an 87% decrease by the eighth week. This meant that fatigue might have occurred and that the best moment to starve should be found out. The findings showed that the established patterns were unpredictable, which supported the theory that the irregular trend in protein production during the fasting phase adhered to a regular pattern (Phull, Dhule, Phull, & Gupta, 2021). There was a variation in the study that showed a relationship between the influence of the starvation period and the quantity of protein (protein concentration) in leech saliva extract. The antibacterial action of medicinal Malaysian CLSE is supported by previous discoveries on the antimicrobial activity of extracts from other leech species, from which various antibacterial peptides were isolated (Alaama et al., 2014, 2024). The results of this investigation shed important light on the dynamic alterations in protein CLSE. During an 8-week fasting duration and the consequences on its antimicrobial effectiveness. It is important to understand the connection between protein concentration and CLSE's antibacterial effectiveness. The greater zones of inhibition seen in our bacterial studies provide evidence that higher protein levels in the 2nd and 4th weeks correlate with increased antibacterial activity. We can conclude that during a prolonged starvation period, more nutrient-dense molecules would break down and be eaten by the leech (Grafskaia et al., 2020).

S. typhimurium, *K. pneumoniae*, and *E. coli* were tested for the antibacterial activities of CLSE. The CLSE obtained during the initial two and four weeks of fasting showed high levels of antibacterial activity against *E. coli*, *S. typhi*, and *K. pneumoniae*. However, the CLSC that was obtained during the eighth week of fasting had decreased antibacterial activity. The antibacterial activity of CLSE is therefore clearly inversely correlated with the duration of hunger, and this correlation has a major effect on the application of CLSE in the management and treatment of certain microbiological illnesses caused by *K. pneumoniae*, *S. typhi*, and *E. coli* (Lazar, Oprea, & Ditu, 2023). The observed patterns highlight the importance of timing in maximizing the antibacterial properties of CLSE. Additionally, the variability among different bacterial strains underscores the need for tailored approaches depending on the target bacteria. Further research is essential to elucidate the mechanisms driving these changes and to optimize the use of CLSE in various applications.

CONCLUSIONS

The Malaysian leech *Hi. manillensis*, researchers have successfully demonstrated in earlier studies that the duration of starvation can impact the production of total protein concentration. This is demonstrated by the safe and ethical collection of CLSE based on that methodology. Because there is variance in the volume difference from the beginning to the end of the study, the difference in the volume output of CLSE according to the set period of time analyzed does not demonstrate significance. Meanwhile, it was shown that leeches create the highest protein concentration during the middle of the hunger period. The study findings demonstrate that leeches can continue to synthesize protein even after their initial harvest. But when comparing the midpoint of the starvation period to the study's beginning, there were no appreciable changes in the generation of total protein concentration. This suggests that, based on the physiological metabolic process of the leech, there is still more protein manufacturing activity taking place. Furthermore, the zone of inhibition observed against CLSE effectively demonstrated the antimicrobial impact and capacity to a group of gram-negative bacteria at the beginning of the fasting period for two weeks. This demonstrates that CLSE can impact particular bacterial species. Consequently, in contrast to studies employing restricted spectrum bacteria, additional research employing a broad range of bacteria is strongly urged.

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دراسة الخصائص المضادة للبكتيريا في لعاب العلق: تأثير الجوع والجرعة على مستخلص لعاب العلق الخام (CLSE) على سلالات البكتيريا

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ملخص

تعتبر علقة الجاموس الآسيوية، *manillensis Hirudinaria*، من الأنواع القيمة في الممارسات الطبية بسبب تشابهها مع علقة الجاموس الأوروبية الطبية. تشتهر العلاج بالعلقات بمزاياها المتعددة، مثل خصائص مضادات التخثر الموجودة في لعاب العلقة والتي تمنع تجلط الدم. درست دراسة تأثير تغذية 60 علقة خلال فترة تجويع مدتها 8 أسابيع على زيادة وزنها وتركيز البروتين في لعابها. أظهرت النتائج أن العلقات شهدت زيادة كبيرة في الوزن في الأسابيع الثانية والرابعة والثامنة، مع تباين في تركيزات البروتين. أظهر استخراج لعاب العلق الخام (CLSE) فعالية مضادة للبكتيريا ضد E. coli و Klebsiella sp. و Salmonella sp.، حيث أظهرت Klebsiella sp. أعلى حساسية. وهذا يشير إلى أن CLSE له تأثير مضاد للبكتيريا يعتمد على الوقت والجرعة، خاصة ضد *Klebsiella sp*. يوصى بإجراء مزيد من الأبحاث لتعزيز فهمنا لتركيز البروتين في لعاب العلق وتحسين CLSE لخصائصه المضادة للبكتيريا.

الكلمات الدالة: *Hirudinaria manillensis*، النشاط المضاد للبكتيريا، استخراج لعاب العلق الخام، الجوع، تركيز البروتين.

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