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The Relative Indirect Anthelmintic Effect of Caprine Milk on Mucins Gene Expression *in Vitro* Using IL-22 Treated LS174T Cells Model of Helminth Infection

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ABSTRACT

Mucus secretion by intestinal goblet cells constitutes an important mechanism in TH2 response following helminth infection mediated by the key cytokine IL-22. This indirect mechanism rather than directly attacking the parasite is important in preventing helminth attachment hence promoting helminth expulsion from the intestinal tract. We hypothesized that natural products having an anthelmintic activity like caprine milk may exert similar response. Using human intestinal LS174T cells treated with IL-22 to simulate helminth infection, we tested whether or not the co-treatment with caprine milk induces MUC1, MUC3, MUC4 and MUC5B genes expression. Optimal concentrations for caprine milk was determined to be 25% and 50% from cell viability assay. IL-22 induced helminth infection model was confirmed. However, the indirect anthelmintic effect of caprine milk was only relative as treatment of caprine milk in LS174T cells and IL-22 *in vitro* did not significantly induce MUC1, MUC3, MUC4 and MUC5B genes expression when compared to treatment with IL-22 alone. In conclusion, caprine milk was not significantly associated with the mechanism of increased mucus production through upregulation of mucin genes by intestinal cells. Caprine milk may possess direct anthelmintic effect rather than indirect.

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INTRODUCTION

Intestinal parasitic helminth infection is a global affliction with significant disease burden (Hotez and Kamath, 2009). In order to survive within the human host, parasitic helminths have evolved various strategies to evade immune attack ranging from passive tactic like using blood group antigens as cover (Dean, 1974) to actively modulating the immune system (Loukas and Prociv, 2001). The body's humoral response to such invasion has been the T-helper type 2 (TH2) response which involves mast cells and various cytokines. With regards to the intestine, such a response involves secretion of mucus to facilitate worm expulsion of weak or dead worms (Grencis *et al.*, 2014). Specific to hyperproduction of mucus are the role of goblet cells and upregulation of mucin genes by IL-13 (Hasnain *et al.*, 2011) and IL-4 (Dabbagh *et al.*, 1999). Interestingly, Turner, Stockinger and Helmby in 2013 had demonstrated the key role of IL-22 in anthelmintic

immunity, particularly in goblet cell function leading to worm expulsion which can be seen as an indirect anthelmintic activity compared to mostly direct effects (Befus, 1977) mounted against worms in the intestine. The control of parasitic helminth infections is principally through chemotherapy (Behnke *et al.*, 2008) but is hampered by the problem of resistance which led many to screen plant based products for anthelmintic activity (Prakash and Mehrotra, 1987).

Animals are also potential sources for medicine. Porcine based therapies like clexane and insulin are hospital mainstays (Teuscher and Berger, 1987). Thrombolytic agents used in stroke like Ancrod was developed from snake venom (Levy *et al.*, 2009). Experimentation on the anthelmintic effects of milk began during the post war era but quickly loses popularity shortly thereafter (Hamdan *et al.*, 2017). Bovine milk exerted an anthelmintic effect causing a reduction in parasites when pigs were given skim milk (Shorb and Spindler, 1947; Spindler *et al.*, 1944; Spindler and Zimmerman, 1944).

Moreover, bovine milk and milk products appear to be active *in vitro* by reducing the motility of both sheathed and exsheathed L3 *Ostertagia* in whey protein (Zeng *et al.*, 2003). The superior anthelmintic activity of camel milk against *Haemonchus contortus* in comparison to milk from cow, ewe and goat were reported *in vitro* (Alimi *et al.*, 2016) and *in vivo* (Alimi *et al.*, 2018) who suspected the role of lactoferrin and vitamin C in mediating the effects. Caprine milk (goat milk), a popular drink in many parts of the world and many cases often being promoted as a functional food with many nutraceutical effects (Najm, 2019).

Evidence of its anthelmintic activity is very limited with (Alimi *et al.*, 2016) documenting a statistically insignificant effect using *in vitro* assay. The mechanism by which caprine milk exerts its anthelmintic activity is poorly understood with only (Najm *et al.*, 2018) observing a direct hydrolysing effect on worm cuticle from transmission electron micrographic study. Would caprine milk affect worms indirectly? We hypothesized that caprine milk may exert an indirect response towards helminth infection inducing the immune system to upregulate mucin genes. Using human Dukes' type B colorectal adenocarcinoma cell line (LS174T) cells treated with IL-22 to simulate helminth infection, we tested whether or not the co-treatment with caprine milk induces mucin 1 (MUC1), mucin 3 (MUC3), mucin 4 (MUC4) and mucin 5B (MUC5B) genes expression by qPCR.

MATERIALS AND METHODS

Milk

Frozen caprine milk was collected from Berkat Jaya farm in Sungai Buloh, Selangor, Malaysia. They were kept at -20°C at Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia (USIM) until use.

Cell culture

The human Dukes' type B colorectal adenocarcinoma (LS174T) (No. C0009013) was purchased from Addexbio (Addexbio, USA). Thawed cells in the vial were transferred into desired culture flask with complete culture Eagle's Minimal Essential Medium (MEM) (Gibco, USA)supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). For passaging adherent cells culture, the culture medium was discarded, and culture flask (Thermo Fisher, USA) was washed using 5 ml (for T75 culture flask) or 10 ml (for T125 culture flask) 1x Phosphate Buffer Saline (PBS) (Gibco, USA). The wash solution was then discarded before pre-warmed dissociation reagent, 0.25% trypsin-EDTA (Gibco, USA) was added and incubated for 4 minutes in 37°C incubators.

Next, the flask was inspected and observed under a light inverted microscope (Olympus CKX 41, Germany) to ensure that cells have completely detached from the flask surface. The same amount of complete culture medium was added into the respective flask to deactivate the trypsin effect. The mixed media with cells was then transferred into 50ml microcentrifuge tube (Eppendorf, USA) and centrifuged at 1,500 x *g* for 7 minutes. The cell pellet was resuspended in a minimal volume of prewarmed complete culture medium and 90μl was aliquoted for cell counting using trypan blue (Sigma-Aldrich, Germany), staining and haemocytometer (Hawksley, England). A total of 5 x 10^5 cells would be an ideal amount for an overnight 80% cell confluence in 6-well plate (Thermo Fisher, USA).

Cell viability assay

The effects of caprine milk on LS174T cell proliferation or inhibition was analyzed using MTS assay kit (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA). Approximately, 1x10^5 cells/well were seeded in 96-well plates and allowed to attach overnight. Cells then were treated with different concentrations based on doubling dilutions (100%, 50%, 25%, 12.5%) of caprine milk and incubated at 37°C in a humidified atmosphere with 5% CO_2 for up to 48 hours.

Following the treatment period, 20μl of MTS reagent

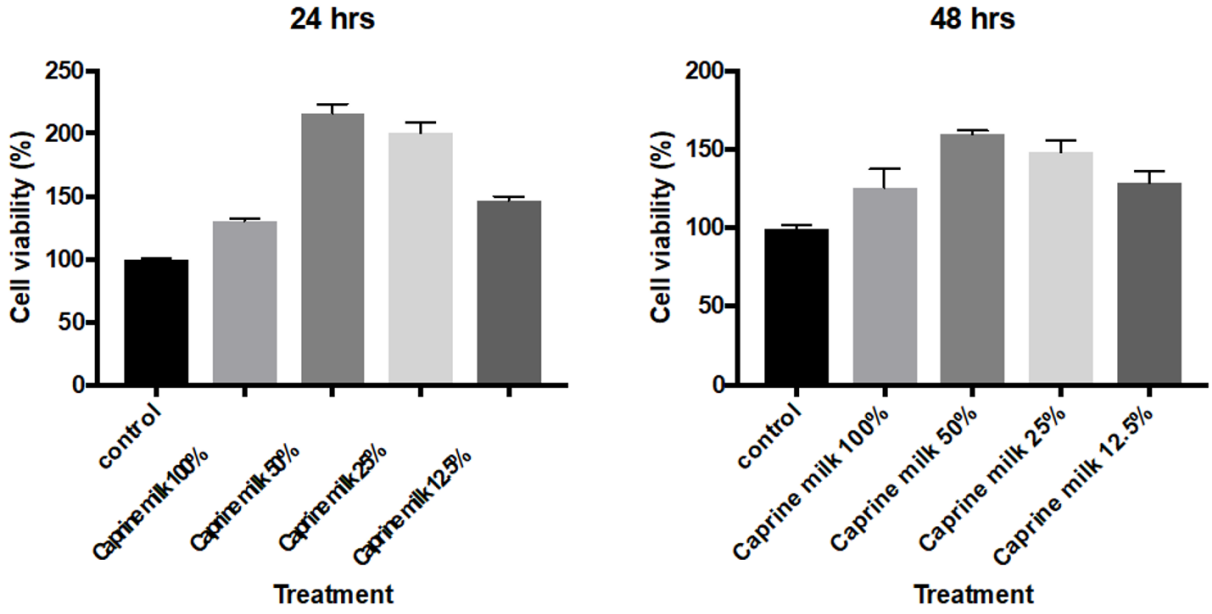

Figure 1: The effect of various concentrations of caprine milk on the rate of LS174T cell proliferation at 24 and 48 hours of incubation.

Table 1: List of forward and reverse primers for mucin 1, mucin 3, mucin 4 and mucin 5B

Gene name	Accession number	Forward primer	Reverse primer
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	99166649	5'-ATCACCA TCTTCCAGGAGCGA-3'	5'-AGCCTTCTCCAT GGTGGTGAA-3'
Mucin 1 (MUC1)	99200183	5'-ACAGTGCTTACA GTTGTTACGGGT-3'	5'-CCTGGCAGAGGT GCCGTTGT-3'
Mucin 3 (MUC3)	99200185	5'-TGGATCTAGATG TAGTGGAGACC-3'	5'-TGCAAAAATCCT CTGCATCTG-3'
Mucin 4 (MUC4)	99200187	5'GCCCAAGCTAC AGTGTGACTCA-3'	5'ATGGTGCCGT TGTAATTTGTTGT-3'
Mucin 5B (MUC5B)	99200181	5'GGGCCTCGAG TGCCGTG-3'	5'CACACGGA TTCATAGTTGAA-3'

(Promega, USA) is added into 100μl of each well containing sample and incubated for 4 hours. Finally, absorbance of each well is recorded at 490nm using microplate reader (Tecan, Switzerland). Percentage of cell viability was calculated using the formula below;

Cell culture model and treatment group

The effects of caprine milk on mucin genes expression of LS174T cells were determined using IL-22-induced LS174T *in vitro* model. The concentration of IL-22 for induction of helminth-like infection condition were optimised. The cell culture model were divided into 4 main groups which are treatment of cells with; (1) growth medium as control 1, (2) IL-22 alone as control 2, (3) caprine milk 50% + IL-22 and (4) caprine milk 25% + IL-22. Each treatment was performed for 24 and 48 hours duration. Upon completion of treatment, cells were harvested for RNA extraction and gene expression analysis.

Quantitative real time PCR (qPCR)

Cells were harvested and stored in TRIzol™ (Invitrogen, USA)reagent at -80°C until processing. RNA was purified using TRIzol™. cDNA was prepared via reverse transcription using SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen, USA) and quantitative PCR was performed using SSO Advanced Universal SYBR Green Supermix (Bio-Rad, USA). Protocols for cDNA synthesis and qPCR were run in Applied Biosystems™ StepOne™ Real-Time PCR System (Thermo Fisher Scientific, USA).

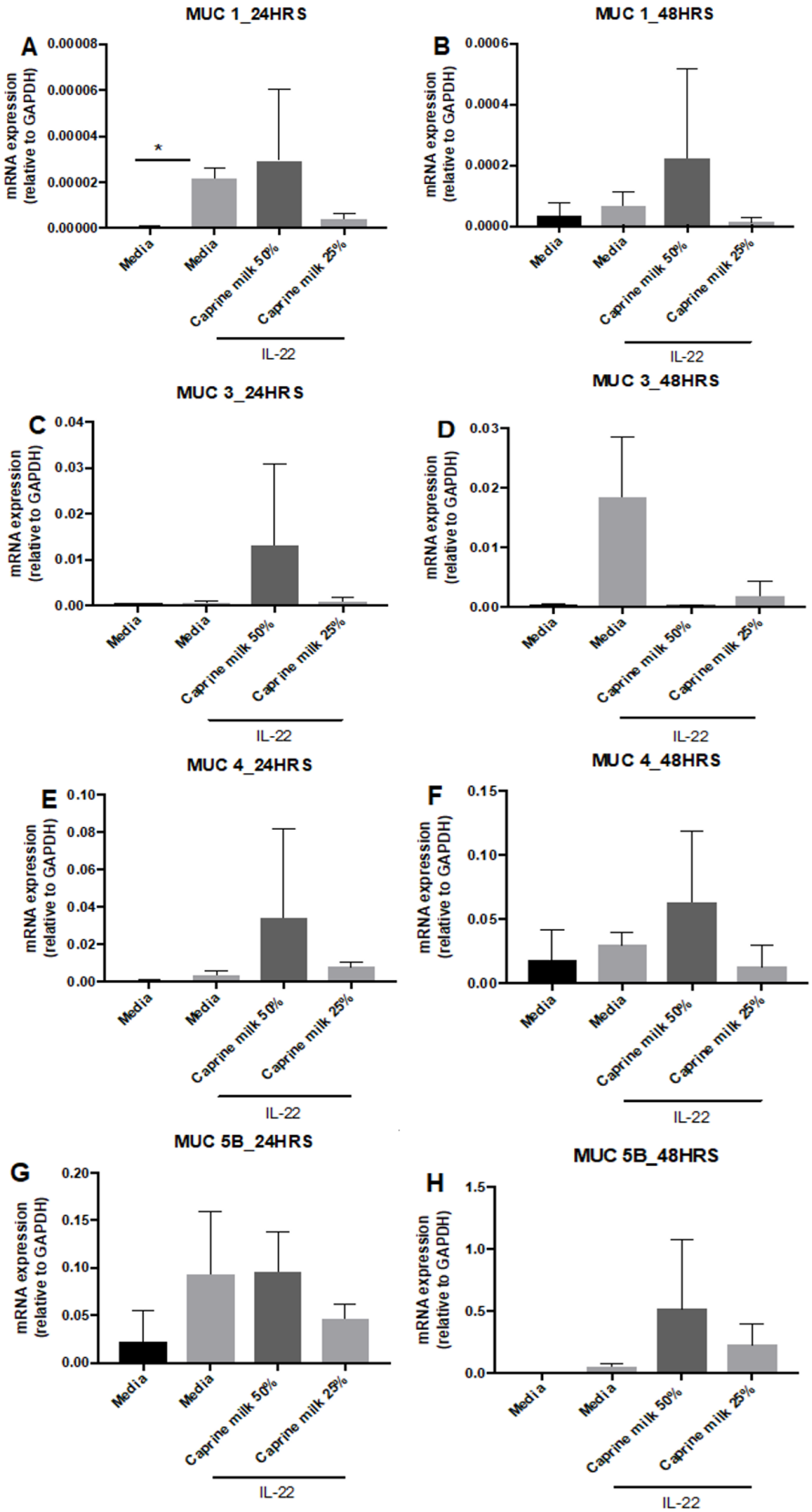

Figure 2: MUC1(A&B); MUC3(C&D); MUC4(E&F); MUC5B(G&H) mRNA expression relative to the housekeeping gene, GAPDH, after 24 and 48 hours of treatment in different concentrations of caprine milk.

All protocols; RNA extraction, cDNA synthesis and qPCR were based on manufacturers' recommendation. Results were normalised to the housekeeping gene GAPDH.

Preparation of forward/reverse primers

The primers for housekeeping gene, GAPDH, and gene of interest; MUC1, MUC3, MUC4 and MUC5B were synthesised based on sequences in Table 1. The primers were reconstituted into 100μM primary stock using 1x TE buffer (pH 8) (Invitrogen, USA) and 100μM stock solution using Nuclease-Free Water (Thermo Fisher Scientific, USA).

Data analysis

Data were analyzed using Graphpad Prism 7 software (USA). Kruskal-Wallis test was used to test significant differences on the expressions of MUC1, MUC3, MUC4 and MUC5B between groups of LS174T cells receiving various concentrations of caprine milk and IL-22. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Cell viability assay

In this study, we tested few concentrations of caprine milk (100%, 50%, 25%, 12.5%) on LS174T cell viability using MTS assay. As shown in Figure 1, LS174T cells remain viable at 24 and 48 hours. From the viability test, 2 concentrations of caprine milk (50%, 25%) showed optimal cells viability hence chosen to proceed with the mRNA expressions of various mucin genes (MUC1, MUC3, MUC4, MUC5B) after treatment of LS174T cells with the cytokine IL-22.

Mucins mRNA expressions after treatment of LS174T cells with caprine milk and IL-22

Expressions of human mucins such as MUC1, MUC3, MUC4 and MUC5B were quantitated using real time qPCR. ΔCt was used to derived $2^{-\Delta Ct}$ based on protocol of Schmittgen & Livak in 2008. There were no significant differences of MUC1, MUC3, MUC4 and MUC5B expressions between LS174T cells treated with IL-22 alone and cells treated with different concentrations of caprine milk (see Figure 2).

DISCUSSION

Our findings failed to observe a significant upregulation of mucin genes when intestinal cells (LS174T) were induced using IL-22 to simulate helminth infection (Turner *et al.*, 2013) and treated with caprine milk which is known to have anthelmintic activity (Alimi *et al.*, 2016; Najm *et al.*, 2018; Najm, 2019). However, cells treated with IL-22 alone successfully upregulated MUC1 expression as compared to cells treated with media only (negative control) confirming the indirect anthelmintic expulsion model proposed by (Turner *et al.*, 2013) as well as strengthening the idea that IL-22 is the key cytokine in such a response. This is important as most work highlighted other TH2 (Zaph *et al.*, 2014; Finkelman *et al.*, 2004) cytokines such as IL-13 (Hasnain *et al.*, 2011) and IL-4 (Dabbagh *et al.*, 1999) which modulates mast cells and macrophages (Allen and Maizels, 2011). IL-22 was also evident in worm therapy experimentation using *Trichuris trichiura* to treat ulcerative colitis patients (Broadhurst *et al.*, 2010).

Our model (LS174T and IL-22) may be used by others who wish to mimic the indirect anthelmintic immune response of helminth infection modelled earlier by (Turner *et al.*, 2013) in which IL-22 alone was able to induce the expression of several goblet cell mediators hence increase mucin production by intestinal epithelium and assists in worm expulsion. However, in the present study we did not include positive control because there is no known substance has been shown to upregulate mucins gene expression. Although not statistically significant, our work demonstrated that caprine milk relatively induced mucin genes expression in a dose dependant manner for MUC1, MUC4 and MUC5B. Most mucins mRNA showed upregulation in at least one concentration of caprine milk treatment.

However, the upregulations were not statistically significant. Hence, there is no significant association of mucin expressions between LS174T cells treated with cytokine IL-22 (control) alone and cells treated with variations of caprine milk concentration. The variations between Ct values of the mucins obtained were quite high. This could contribute to the insignificant data results from this qPCR analysis. However, the trend of increased mucins gene expression from caprine milk treatment is noted and should be evaluated further.

CONCLUSION

Human LS174T intestinal cells treated with IL-22 and caprine milk *in vitro* did not induce MUC1, MUC3, MUC4 and MUC5B genes expression. Therefore, the anthelmintic effect of caprine milk is not associated with indirect effects of intestinal cells increasing mucus production through upregulation of mucin genes. Caprine milk may be exerting its anthelmintic effect directly onto the helminth cuticle proteolytically digesting them much like cysteine proteinases.

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Conflict of interest

The authors declare that they have no conflict of interest for this study.

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REFERENCES

Alimi, D., Abidi, A., Sebai, E., Rekik, M., Maizels, R. M., Dhibi, M., Akkari, H. 2018. In vivo nematicidal potential of camel milk on Heligmosomoides polygyrus gastro-intestinal nematode of rodents. *Helminthologia*, 55(2):112–118.

Alimi, D., Hajaji, S., Rekik, M., Abidi, A., Gharbi, M., Akkari, H. 2016. First report of the in vitro nematicidal effects of camel milk. *Veterinary Parasitology*, 228:153–159.

Allen, J. E., Maizels, R. M. 2011. Diversity and dialogue in immunity to helminths. *Nature Reviews Immunology*, 11(6):375–388.

Befus, A. D. 1977. Hymenolepis diminuta and H. microstoma: Mouse immunoglobulins binding to the tegumental surface. *Experimental Parasitology*, 41(1):242–251.

Behnke, J. M., Buttle, D. J., Stepek, G., Lowe, A., Duce, I. R. 2008. Developing novel anthelmintics from plant cysteine proteinases. *Parasites & Vectors*, 1(1):29–29.

Broadhurst, M. J., Leung, J. M., Kashyap, V., McCune, J. M., Mahadevan, U., McKerrow, J. H., Loke, P. 2010. IL-22+ CD4+ T Cells Are Associated with Therapeutic Trichuris trichiura Infection in an Ulcerative Colitis Patient. *Science Translational Medicine*, 2(60):60ra88–60ra88.

Dabbagh, K., Takeyama, K., Lee, H. M., Ueki, I. F., Lausier, J. A., Nadel, J. A. 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *The Journal of Immunology*, 162(10):6233–6237.

Dean, D. A. 1974. Schistosoma mansoni: adsorption of human blood group A and B antigens by schistosomula. *The Journal of parasitology*, pages 260–263.

Finkelman, F. D., Shea-Donohue, T., Morris, S. C., Gildea, L., Strait, R., Madden, K. B., Schopf, L., Urban, J. F. 2004. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunological Reviews*, 201(1):139–155.

Grencis, R. K., Humphreys, N. E., Bancroft, A. J. 2014. Immunity to gastrointestinal nematodes: mechanisms and myths. *Immunological Reviews*, 260(1):183–205.

Hamdan, F., Addenan, F. H., Manzor, N. F. M., Abdullah, W. O., Elkadi, M. A., Rauf, A. N. A., Aripin, K. N. N., Mansur, F. A. F. 2017. A Systematic Review on the Anthelmintic Effects of Milk. *Advanced Science Letters*, 23(5):4993–4996.

Hasnain, S. Z., Thornton, D. J., Grencis, R. K. 2011. Changes in the mucosal barrier during acute and chronic Trichuris muris infection. *Parasite Immunology*, 33(1):45–55.

Hotez, P. J., Kamath, A. 2009. Neglected Tropical Diseases in Sub-Saharan Africa: Review of Their Prevalence, Distribution, and Disease Burden. *PLoS Neglected Tropical Diseases*, 3(8):e412–e412.

Levy, D. E., Zoppo, G. J. D., Demaerschalk, B. M., Demchuk, A. M., Diener, H. C., Howard, G., Wasiewski, W. W. 2009. Ancrod in acute ischemic stroke: results of 500 subjects beginning treatment within 6 hours of stroke onset in the ancrod stroke program. *Stroke*, 40(12):3796–3803.

Loukas, A., Prociv, P. 2001. Immune Responses in Hookworm Infections. *Clinical Microbiology Reviews*, 14(4):689–703.

Najm, O. A. 2019. Anthelmintic effects of prophetic foods (Ajwa date fruit and goat's milk) on gastrointestinal helminths, Doctoral thesis. Universiti Sains Islam Malaysia.

Najm, O. A., Addnan, Fh, Elkadi, Ma, Abdullah, Wo, Manzor, Nfm, Abidin, Mansur, F. 2018. Anthelmintic activity of goat's milk: Transmission electron micrographic evidence. *Scholars International Journal of Chemistry and Material Sciences*, 1(3):77–81.

Prakash, V., Mehrotra, B. N. 1987. Anthelmintic plants in traditional remedies in India. *Indian Journal of history of Science*, 22(4):332–340.

Shorb, D. A., Spindler, L. A. 1947. Growth rate of pigs fed skim milk to control intestinal parasites. *Proceedings of the Helminthological Society of Washington*, 14(1):30–34.

Spindler, L. A., Zimmerman, H. E. 1944. Effect of skim milk on the growth and acquisition of parasites by pigs under conditions of constant exposure to infection. *Proceedings of the Helminthological Society of Washington*, 11(2):49–54.

Spindler, L. A., Zimmerman, H. E., Hill, C. H. 1944. Preliminary observations of the control of worm parasites in swine by the use of skim milk. *Proceedings of the Helminthological Society of Washington*, 11(1):9–12.

Teuscher, A., Berger, W. G. 1987. Hypoglycaemia unawareness in diabetics transferred from beef/porcine insulin to human insulin. *The Lancet*, 330(8555):382–385.

Turner, J.-E., Stockinger, B., Helmby, H. 2013. IL-22 Mediates Goblet Cell Hyperplasia and Worm Expulsion in Intestinal Helminth Infection. *PLoS Pathogens*, 9(10):e1003698–e1003698.

Zaph, C., Cooper, P. J., Harris, N. L. 2014. Mucosal immune responses following intestinal nematode infection. *Parasite Immunology*, 36(9):439–452.

Zeng, S., Brown, S., Przemeck, S. M. C., Simpson, H. V. 2003. Milk and milk components reduce the motility ofOstertagia circumcinctalarvaein vitro. *New Zealand Veterinary Journal*, 51(4):174–178.