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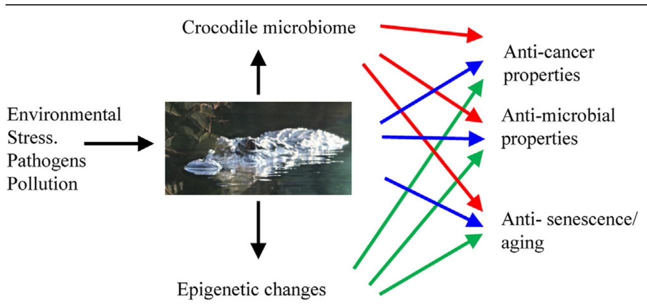
Review article

Longevity, cellular senescence and the gut microbiome: lessons to be learned from crocodiles

Ruqaiyyah Siddiqui [a], Sutherland Maciver [b], Adel Elmoselhi [c], Nelson Cruz Soares [d,e], Naveed Ahmed Khan [c,*]

[a] College of Arts and Sciences, American University of Sharjah, University City, Sharjah 26666, United Arab Emirates
[b] Centre for Discovery Brain Sciences, Edinburgh Medical School: Biomedical Sciences, University of Edinburgh, Edinburgh, UK
[c] College of Medicine, University of Sharjah, Sharjah, 27272, United Arab Emirates
[d] College of Pharmacy, University of Sharjah, Sharjah, 27272, United Arab Emirates
[e] Sharjah Institute for Medical Research, University of Sharjah, Sharjah, 27272, United Arab Emirates

GRAPHICAL ABSTRACT



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ABSTRACT

Crocodiles are flourishing large-bodied ectotherms in a world dominated by endotherms. They survived the Cretaceous extinction event, that eradicated the dinosaurs who are thought to be their ancestral hosts. Crocodiles reside in polluted environments; and often inhabit water which contains heavy metals; frequent exposure to radiation; feed on rotten meat and considered as one of the hardy species that has successfully survived on this planet for millions of years. Another capability that crocodiles possess is their longevity. Crocodiles live much longer than similar-sized land mammals, sometimes living up to 100 years. But how do they withstand such harsh conditions that are detrimental to *Homo sapiens*? Given the importance of gut microbiome on its' host physiology, we postulate that the crocodile gut microbiome and/or its' metabolites produce substances contributing to their "hardiness" and longevity. Thus, we accomplished literature search in PubMed, Web of Science and Google Scholar and herein, we discuss the composition of the crocodile gut microbiome, longevity and cellular senescence in crocodiles, their resistance to infectious diseases and cancer, and our current knowledge of the genome and epigenome of these remarkable species. Furthermore, preliminary studies that demonstrate the remarkable properties of crocodile gut microbial flora are discussed. Given the profound role of the gut microbiome in the health of its' host, it is likely that the crocodile gut microbiome and its' metabolites may be contributing to their extended life expectancy and elucidating the underlying mechanisms and properties of these metabolites may hold clues to developing new treatments for age-related diseases for the benefit of *Homo sapiens*.

* Corresponding author.
E-mail address: naveed5438@gmail.com (N.A. Khan).

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1. Introduction

Crocodiles are a remarkable species, being designated as "living fossils" and have been described as anachronism (Meyer, 1984; Stockdale and Benton, 2021). They have been around for the last 85 million years, and survived mass extinction events, such as the catastrophic Cretaceous-Tertiary extinction event (Tokaryk, 1991; Jeyamogan et al., 2020). In fact, ancestors of the crocodile have existed since the Early Jurassic period of around 200 Million years ago, and looked very similar to the modern crocodiles (Tykoski et al., 2002; Stockdale and Benton, 2021). *Homo sapiens* on the other hand, are just one species amongst millions of other species, being a recent addition to the planet Earth. Crocodiles have shown the ability to adapt, evolve and survive successfully over millions of years, suggesting that we ought to learn from these species. A recent study states that crocodiles followed a state of "punctuated equilibrium" in the evolutionary process, by reaching an optimum state and remaining as such, until the environment forces them to adapt to new conditions (Stockdale and Benton, 2021). Currently, there are 24 species of crocodile, whereas other species such as birds have achieved a diversity of many thousands of species over a similar period of time, suggesting the robustness of this strategy (Grigg, 2015). Interestingly, crocodiles have been utilized in traditional Chinese medicine dating back to the 16th century Ming Dynasty. The use of crocodile products is well recognized in traditional Chinese medicine, with the use of crocodile bile or dried crocodile meat as an important therapeutic regimen (Chui et al., 2006; Ziment and Tashkin., 2000; Ng et al., 2003). In China, crocodile meat is thought to strengthen the body, promote longevity, relieve asthma, and help with a plethora of other ailments (Zhang et al., 2021; Williams et al., 2016). Several lines of evidence have highlighted the role of the gut microbiome in its' host health such as immunity, metabolism, nervous system development and disorder, protection against infectious diseases, endocrine pathways among other physiological processes (Siddiqui et al., 2021; Valdes et al., 2018). Hence it is logical to propose the role of crocodile gut microbiome in contributing to its health and resilience. Herein we describe the crocodile gut microbiome composition, in relation to crocodile longevity and cellular senescence, the genome and epigenome as well as the resistance of crocodiles to infectious diseases and cancer. Accordingly, literature search was performed until 31 May 2021, in PubMed, Web of Science and Google Scholar and only articles published in the English language were included. Preliminary studies investigating the anti-cancer and anti-bacterial effects of crocodile gut bacterial metabolites and the molecules elucidated are also discussed. Moreover, we deliberate the role of the crocodile gut microbiome in the "hardiness" of these remarkable species.

2. Crocodile gut microbiome composition and novel metabolites

The gut microbiome is now well known to play a profound role on the health of the host and a plethora of studies in humans have shown that the gut microbiome can provide protection against neurodevelopment disorders, as well as cancer (Siddiqui et al., 2021). However, there is a dearth of studies on determining the gut microbiome composition of reptiles. To date there has been just two published studies which determined the gut microbiome composition of the Australian saltwater crocodile (*Crocodylus porosus*) (Willson et al., 2019), along with another study which examined the gut bacteria of the salt water crocodile (*Crocodylus porosus*) in Malaysia (Khan et al., 2021). The study on the Australian saltwater crocodile revealed that the gut microbiome was dominated by *Firmicutes*, mainly *Clostridia,* and *Fusobacteria*, indicating that the crocodile gut microbiome is indeed distinct from mammals, fish, and other reptiles which are generally dominated by *Firmicutes* and *Bacteroidetes* (Willson et al., 2019). Interestingly, a high abundance of pathogenic bacterial species were observed, this was in agreement with a prior study conducted on alligators as well as another study on crocodile lizards (Keenan et al., 2013; Tang et al., 2020). Furthermore, the presence of *Fusobacteria* in the gut microbiome of alligators made the alligator gut microbiome unique (Keenan et al., 2013). It was revealed that the core microbiota of the crocodile was specifically different to the mammal gut microbiome as well as fish and other reptiles. Unlike assemblages of phyla generally represented by higher abundances of *Firmicutes* and *Bacteroidetes*, the alligator microbiota was dominated by *Firmicutes* and *Fusobacteria*.

As studies in *Homo sapiens* have shown that in order to prevent and even treat many lifestyle diseases that increase with age, the gut microbiome is of utmost importance (Valdes et al., 2018), in this regard the investigation of the plethora of microbial species and the molecules they produce, as well as the genomes in the crocodile gut microbiome are warranted. In a recent study, bacteria were isolated from different locations of the gastrointestinal tract of the saltwater crocodile (*Crocodylus porosus*), namely the mouth and esophagus, stomach, large and small intestines, anus and 16S rDNA sequencing was accomplished. Bacterial species comprising, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Deinococcus-Thermus* were found to inhabit the crocodile gut, and conditioned media containing the bacterial metabolites were prepared (Khan et al., 2021). These metabolites were then evaluated against cancer cells *in vitro* and metabolites which depicted inhibition of cell metabolic activity or viability reduction, and cell survival inhibition were observed. Furthermore, liquid chromatography-mass spectrometry (LC-MS) was performed to elucidate the nature of molecules in the crocodile gut bacterial metabolites with the analysis portraying numerous molecules, albeit many remained unidentified and thus may be potentially novel. The LC-MS data revealed that bacterial metabolites from the duodenum of *C. porosus* contained a flavonoid which has previously reported anticancer activity, known as; l,l-Cyclo(leucylprolyl). l, l-cyclo(leucylprolyl). Other molecules identified include those with antibacterial activity, namely (lactic acid, f-Honaucin A, l, l-Cyclo(leucylprolyl), antifungal activity [3-hydroxy-decanoic acid), however, the precise nature of these molecules remains unidentified (Khan et al., 2021). These are the only studies that have been conducted to elucidate the gut microbial metabolites of crocodiles, based on our knowledge. Given that these species are so resilient, as well as long lived and display limited cellular senescence (Jones et al., 2014) the ability of crocodiles to adapt to their environment, their resistance to infection and cancer is most likely associated with their gut microbiome, as it is thought that 70–80% of immune cells are present in the gut, portraying an intricate relationship with the gut microbiome (Wiertsema et al., 2021). These gut bacterial metabolites should be tested in models of ageing such as the heterogeneous mouse model (HET) developed by the National Institute on Aging interventions testing program to explore their effects as well as determine their mechanism of action (Folch et al., 2018). Furthermore, invertebrate models such as yeast and *C. elegans* may be utilized, as they have been used to portray that affect aging such as insulin signalling and mitochondrial alteration, sirtuins, caloric restriction in prior studies and have been originally and extensively investigated in these models (Folch et al., 2018). An alternate strategy could be implantation of selected gut microbial metabolites of crocodiles into models of ageing or disease, however intensive further research will be required. It is hoped that such studies will identify several molecules that can be later utilized in humans.

3. Longevity of crocodiles and cellular senescence

Ectothermic reptiles such as the crocodiles depict various features that deem them well suitable to comprehend the evolution of ageing. These comprise factors such as patterns of indeterminate growth and increased fecundity with age, as well as their physiology is adapted to endure stressful environments throughout the course of life (Briggs-Gonzalez et al., 2017).

Cellular senescence is the process of disintegration of physiological and biochemical function with increasing age, thus leading to age-related issues and functional deterioration. At the level of the cell, senescence involves complex cellular signalling pathways, functional and genetic changes (Hoekstra et al., 2020; Van Deursen, 2014). Furthermore, cellular senescence has been attributed to the prevention of carcinogenesis, and more recently, aging, development, and tissue repair. It is thought that cellular senescence offers substantial benefit to the host via induction of irreversible cell cycle arrest, as well as activation of immune-mediated clearance of tumor cells, characterized by reduced incidence of cancer (Yang et al., 2021). Nevertheless, this state of stable proliferative arrest is associated with the senescence-associated secretory phenotype (SASP), that may contribute to age-related conditions, as well as cancer (Cuollo et al., 2020). However, senescent cells and SASP components may directly or indirectly promote tumor cell growth, invasion and metastasis, and tumor vascularization. However, these mechanisms are not yet understood in crocodiles and may be an important contributory factor in the lack of cancer cases in crocodiles (Jeyamogan et al., 2017). Crocodiles possess traits that are linked with decreased mortality and may also be potentially connected with reduced rates of senescence. For instance, reptiles such as crocodiles possess "protective phenotypes" or adaptations that may impact their mortality (Hoekstra et al., 2020). These protective phenotypes in reptiles comprise venom or toxicity in lizards or snakes, skin armour in crocodilians, and external ribcages or shells in turtles (Hoekstra et al., 2020). Furthermore, studies depict that extreme longevity as well as negligible senescence are seen in reptiles (de Magalhaes and Costa, 2009). Recently a study revealed slow or negligible senescence in relation to mortality in three reptiles, namely the freshwater crocodile (*Crocodylus johnstoni*), desert tortoise (*Gopherus agassizi*) and lizard (*Zootoca vivipara*) and showed slow or negative rates of senescence with respect to mortality (Jones et al., 2013). Interestingly, both the crocodile and desert tortoise depicted no decline in reproduction even with their advancing age.

Recently, studies have shown that the gut microbiome composition is linked to various conditions of aging in humans, comprising cardiovascular diseases, cancer, dementia, and other age related and inflammatory mechanisms that may contribute to the accumulation of aged immune cells, metabolic dysregulation, and oxidative stress-mediated macromolecular damage (Renson et al., 2020). Various work has shown that the development of age-related diseases is connected with dysbiosis of the gut (Narasimhan et al., 2021). Furthermore, it is now understood that aging is usually connected with decreased diversity of the gut microbiome, in particular the *Firmicutes* to *Bacteroides* ratio, as well as elevation in some opportunistic species and some *Proteobacteria* (Biagi et al., 2010). As indicated above, it seems that reptiles display negligible senescence and continue to reproduce in their advanced age, and this is most likely due to the nature of their gut microbiome. Moreover, studies to understand the role of cellular senescence in crocodiles as well as the connection with crocodile immune system and gut microbiome is warranted.

4. The genome and epigenome of crocodiles

Comprehension of both the genome and epigenome of crocodilians and in particular crocodiles will shed light on the adaptive abilities as well as the genetic diversity of these remarkable species. Interestingly, analyses of the crocodilian genome depicted that they have evolved very slowly over the past several million years, and it will be useful to grasp how slow-evolving species such as the crocodile are able to thrive over millions of years despite the ongoing environmental changes (Green et al., 2014). Recently, a *de novo* genome assembly of the saltwater crocodile, *Crocodylus porosus*, was generated. The high-quality assembled genome was 2,123.5 Mb. Moreover, 23,128 genes as well as 4,258 pseudogenes were annotated and recognized, further augmenting previous studies. Augmentation of particular genes involved in certain metabolic and cellular mechanisms, for instance potassium channels pore and peroxisomal membrane proteins were noted, which relate to the saline environment that *C. porosus* live in (Ghosh et al., 2020). This newly generated genome will be a valuable resource to investigate the genetic link with longevity, aging and resilience of crocodiles.

The epigenome is the record of DNA changes in the organism and comprises of the various environmental changes over lifespan, thus is of interest (Pal and Tyler, 2016). Many epigenetic events are associated with ageing, however how these events vary in different species is not well understood (Paoli-Iseppi et al., 2017). Moreover, the genome and the environment of the species are thought to impact the etiology of disorders, longevity, and overall health with epigenetic mechanisms as key mediators in these complex interactions (Liu et al., 2008). Many studies related to global DNA methylation, conducted in humans have linked methylation to cancer incidence and other diseases as well exposure to environmental toxins environmental exposures, the presence of cancer, and other diseases (Choi et al., 2009). Nonetheless, limited studies regarding the epigenome of reptiles have been conducted. In fact, alterations in the epigenome will be useful to epigenetic biomarkers to assess the environmental exposures and the impact on reptile health. Interestingly, a study was conducted in alligators residing three different lakes in Florida, which comprised different levels of contaminants. The results from the study indicated that there was no significant difference in DNA methylation from the three different groups, showing the resilience of these species against environmental contaminants (Guillette et al., 2016). Another earlier study from the same group that examined methylation in alligators that were raised under controlled environments in comparison with alligators with varying environmental conditions. The study did not detect any alteration to global DNA methylation implying that the varying environmental parameters did not alter global DNA methylation (Parrott et al., 2014). Current knowledge regarding DNA methylation in animals is on the rise, however data on DNA methylation in reptiles is very scarce. The basic patterns of DNA methylation appear to be similar to those seen in other vertebrates, for instance the depiction of non-methylated islands in gene promoters (Long et al., 2013). Nonetheless, more work needs to be done to understand reptilian epigenetic mechanisms. Epigenetic therapy, often in combination with other therapies, may become a potent tool to treat various diseases that comprise, heart disease, diabetes, and that are impacted by these changes. Given, the resilience of crocodiles and their longevity, studies on the genome and epigenome of these species are warranted using high throughput multi-OMICS technologies in the investigation of the crocodile microbiome. In this regard, multi-omics technologies are now available to mine the microbiome, including transcriptomics/16S ribosomal sequencing, and to gain an in depth insight into the functional microbial communities in human or animal gut microbiome, hence metagenomics data would be complemented with meta-transcriptomics analysis and coupled with mass spectrometry and nuclear magnetic resonance can aim at metaproteomic, proteomgenomics and meta-metabolomic profiles of the constituent microbial communities as well as host cells (Nyholm et al., 2020).

5. Crocodile resistance to infectious agents

Crocodiles are a territorial species and are injured frequently during aggressive episodes; both intraspecies and interspecies, often leading to serious injuries that may even involve the loss of limbs (Merchant et al., 2006). Nonetheless, despite residing in habitats that contain many pathogenic microorganisms, there is often wound-healing in these animals without obvious infections. Several species of crocodilians have been shown to be resistant to infectious agents. For example, it was found that there is a high incidence of *Salmonella* in farmed *Crocodylus porosus* and *Crocodylus johnsoni* (Manolis et al., 1991). In another study, a plethora of *Salmonella* serotypes were observed in healthy Nile crocodiles (*Crocodylus niloticus*) (Madsen et al., 1998).

Various work has shown that serum from crocodilians possess antimicrobial properties. A recent study depicted the anti-amoebic properties of crocodile sera from the mugger crocodile (*Crocodylus palustris*). It was found that crocodile sera abolished the viability of *Acanthamoeba*, a free-living amoebae and opportunistic pathogen. In addition, lysates from various tissues of the crocodile depicted potent anti-amoebic activity

(Siddiqui et al., 2017). In another study, it was shown that crude extracts from the tissues of the Nile crocodile (*Crocodylus niloticus*) exhibited antimicrobial activity (Shaharabany et al., 1999). Furthermore, recent work on the fresh water crocodile (*Crocodylus siamensis*) was carried out, and an antibacterial compound from blood was partially purified and functionally characterized and named as crocosin (Preecharram et al., 2010). Crocosin was shown to exhibit activities against *Salmonella typhi* and *Staphylococcus aureus*. It was speculated that this compound might be used as a defense mechanism from bacterial infections in freshwater (Preecharram et al., 2010).

This work was corroborated further by other studies in the close relative: the American alligator (*Alligator mississippiensis*) which was shown to exhibit potent antibacterial, antiviral and amoebicidal efficacies (Merchant et al., 2003, 2004, 2005). Moreover, in another recent study four novel antibacterial peptides were elucidated from Siamese crocodile white blood cell extracts termed as leucrocins (Pata et al., 2011). These exhibited potent antibacterial efficacies against *Vibrio cholerae*, *Staphylococcus epidermidis*, and *Salmonella typhi* (Pata et al., 2011). Future studies are warranted to determine the precise nature of these antimicrobial effects; as well as the link with the gut microbiome, given that the gut microbiome plays a profound role in the functioning of the immune system.

6. Crocodile anti-cancer properties and ageing

Both cancer and aging are interrelated biologically (Fane and Weeraratna, 2020). It is now understood that cellular processes for instance response to DNA damage as well as cellular senescence are involved in suppressing tumors as well as contributing to ageing in general (Di Micco et al., 2021). Moreover, increasing age in humans is considered a risk factor for cancer development (Seigel et al., 2015). Nonetheless, the connections between aging and cancer are complex as aging comprises loss of organ function as well as tissue degeneration, whereas cancer is a state of sustained cellular proliferation and gain of new functions (Zinger et al., 2017).

Recently, we speculated that animals such as crocodiles possess anticancer properties. This was based on the longevity of crocodiles, with some living up to 100 years and our visits to numerous crocodile farms and sanctuaries as well as personal communication with veterinarians and zoologists, who indicated the absence of cancer cases in these species (Jeyamogan et al., 2017). In this regard a study was conducted, whereby the anti-cancer effects of crocodile sera and various organ lysates were investigated against cervical, breast and prostate cancer cells (Jeyamogan et al., 2020). Crocodile serum depicted potent growth inhibitory effects as well as cytotoxic effects against the cancer cells investigated. Moreover, 80 molecules were elucidated as well as 19 molecules were identified from the crocodile. In addition, approximately 100 potential anticancer peptides were recognized from crocodile serum via a bioinformatics approach centered on binary profile, dipeptide and peptide amino acid composition, and pseudo-amino acid composition (Jeyamogan et al., 2020). Moreover, this study elucidated the differential gene analysis of cancer cells incubated with crocodile sera. These analyses revealed that 51 genes in breast cancer cells, 14 genes in treated cervical cancer cells and 2 genes in prostate cancer cell lines were differentially expressed in comparison to untreated cells and the study revealed that these genes were typically involved in cellular communication, DNA growth and repair, respiration and others (Jeyamogan et al., 2020). Nonetheless, the precise genetic mechanisms for these anti-cancer effects remain to be determined, and further research to determine the connection with the gut microbiome and its' metabolites are currently underway (Khan et al., 2021). It will be interesting to unravel which precise gut molecules exert these potent anti-cancer effects and to develop these into pro/prebiotics for human consumption in the treatment and prevention of cancer. Furthermore, the various hallmarks of ageing, in particular epigenetic mechanisms, genomic instability, telomere attrition, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication need to be explored in crocodiles and reptiles.

7. Concluding remarks and future perspectives

Ageing is arguably one of the key biological challenges affecting human health, thus comprehending the biological mechanisms of senescence and how disease etiologies come about will be of immense value. Furthermore, with an increase in the ageing population worldwide, research on longevity is now an enticing market for the biotech industry (de Magalhaães, 2021). In reptiles such as crocodiles, aging can be limited and their longevity is possibly extended, which is of enormous commercial value if this can be translated for the benefit of *Homo sapiens*. At present there is limited understanding of the crocodile gut microbiome and for reptiles in general, although preliminary studies have isolated and identified potentially innovative as well as some already known molecules from crocodile gut bacterial metabolites which depict inhibition of cell metabolic activity or viability reduction, and cell survival inhibition in cancerous cell lines (Khan et al., 2021). Furthermore, crocodile serum portrays powerful growth inhibitory effects as well as cytotoxic effects against cancer cells, and several known and unknown molecules were identified from the crocodile sera, as well as and estimated 100 potential anticancer peptides being recognized (Jeyamogan et al., 2020). Further work on the microbiome and its' associated metabolites may lead to the recognition of potential innovative therapeutic leads for clinical and pre-clinical investigations in invertebrate and animal models of ageing and/or disease (Figure 1). Alternatively, direct implantation of select gut microbiome species into mammalian models of disease or ageing could be accomplished, leading to recognition of various therapeutic interventions for the benefit to human health.

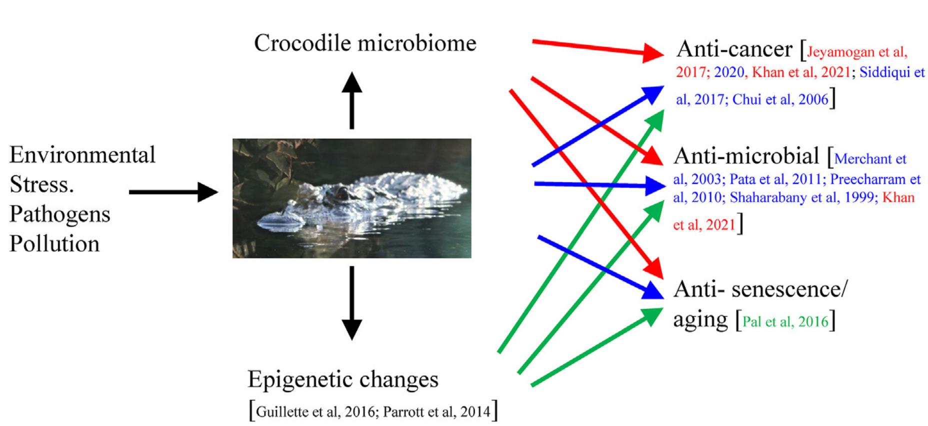

Figure 1. Crocodile microbiota may offer a tremendous source of novel metabolites to counter communicable and non-communicable hazards to human health.

Although these notions may seem far-fetched, other studies such as the discovery of insulin was made when it was found that an aqueous pancreatic extract was able to normalize diabetes in a dog. Previously, insulin for clinical use was normally obtained from cows and pigs (Kehoe, 1989; Crasto et al., 2016). In another example, obesity was elevated with an increase in *Firmicutes* in a mouse model (Turnbaugh et al., 2008). Such studies could be emulated to utilize the unique microbiome of crocodiles for the benefit of *Homo sapiens* with *in vivo* work and clinical trials in the prospective years. However, there are various challenges that a mechanistic-level study in crocodilians presents, such as the difficulty in obtaining permission and access to work with these species, and the logisitics in obtaining gut bacterial samples to ensure reproducibility. Furthermore, immune system mediated responses such as T cell proliferation and allograft rejection are affected significantly by the season, thus this needs to be taken into account when obtaining gut microbial samples or studying the robust crocodile immune system (Zimmerman et al., 2010; Zimmerman, 2020). Being one of the most successful and diverse animal groups in evolution, the gut microbiome of crocodiles may offer a worthy resource for novel bioactive molecules of therapeutic potential.

Declarations

Author contribution statement

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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