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COMBATTING METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) IN THE FOOD INDUSTRY BY HARNESSING THE POWER OF NATURE: A SYSTEMATIC REVIEW

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Abstract

Antibiotic resistance is a critical global health concern, with *Methicillin-resistant Staphylococcus aureus (MRSA)* posing a significant challenge due to its resistance to commonly used antibiotics. Recent research has revealed the potential of natural compounds and microorganisms in combatting *MRSA* and other antibiotic-resistant bacteria. In this systematic review, we studied the effect of essential oils, bacteriophages, bacteriocins, and probiotics on *S. aureus,* including *MRSA* in particular, in the food industry. Essential oils (EOs) have gained significant attention because of their antimicrobial properties, inhibiting *MRSA* growth by damaging bacterial cells and inhibiting essential enzymes and compounds. Cinnamon oil liposomes caused the most significant decrease in *MRSA* populations among our reviewed essential oils. Bacteriophages can lyse the bacterial host. They encode peptidoglycan hydrolases called endolysins that target the bacterial cell wall. In our study, *S. aureus* phage (containing *CHAPLysGH15* and *LysGH15*), and phage *SA11* endolysin *(LysSA11*) were the most effective against *S. aureus*. Bacteriocins, antimicrobial peptides produced by bacteria, also show potential in combatting *MRSA*, mainly by generating organic acids that interfere with bacterial metabolism. According to our review, the

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most effective bacteriocins against *S. aureus* were *Enterocin AS-48* with phenolic compounds or with *2NPOH*, Bacteriocin isolated from *Lactobacillus pentosus - Pentocin JL-1*, and bacteriocin produced by *S. pasteuri RSP-1,* respectively. Probiotics can compete with pathogens by producing antimicrobial compounds that disrupt *MRSA* cell production and ultimately lead to bacterial death. In our review, the most effective probiotics were *Streptomyces griseus*, *Pediococcus acidilactici strains A11 and C12*, *Lactococcus lactis*, and *Lactobionic acid* respectively. A multi-hurdle approach combining these natural agents has shown promising results in targeting and eliminating *MRSA* cells. By harnessing the power of nature, we can potentially overcome the challenges posed by *MRSA* and other antibiotic-resistant bacteria.

Key words: *Methicillin-resistant Staphylococcus aureus (MRSA)*, Essential oils, Bacteriophage, Bacteriocin, Probiotic

BORBA PROTIV STAPHILOCOCCUS AUREUS-a (MRSA) OTPORNOG NA METICILIN U PREHRAMBENOJ INDUSTRIJI KORIŠĆENJEM SNAGE PRIRODE: SISTEMATSKI PREGLEDNI RAD

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Kratak sadržaj

Otpornost na antibiotike je globalni zdravstveni problem, a meticilin rezistentan *Staphilococcus aureus* (MRSA) predstavlja značajan izazov zbog otpornosti na antibiotike koji se obično koriste. Skorija istraživanja otkrila su potencijal prirodnih jedinjenja i mikroorganizama u borbi protiv MRSA i drugih bakterija otpornih na antibiotike. U ovom preglednom radu proučavan je efekat eteričnih ulja, bakteriofaga, bakteriocina i probiotika na *S. aureus*, uključujući i izolate MRSA, u prehrambenoj industriji. Eterična ulja (EO) privukla su značajnu pažnju zbog svojih antimikrobnih svojstava, inhibirajući rast MRSA tako što oštećuju bakterijsku ćeliju i inhibiraju njihove esencijalne enzime i jedinjenja. Od svih ispitanih eteričnih ulja, lipo-

zomi ulja cimeta doveli su do najznačajnijeg smanjenja populacije MRSA. Bakteriofagi mogu da liziraju bakteriju koju napadaju. Oni sintetišu enzime peptiodoglikan hidrolaze koji su poznati pod nazivom - endolizini, koji oštećuju bakterijski zid. U našoj studiji, *S. aureus* fage (koje sadrže CHAPLisGH15 i LisGH15) i fag SA11 endolizin (LisSA11) bili su najefikasniji protiv *S. aureus*. Bakteriocini, antimikrobni peptidi koje proizvode bakterije, takođe pokazuju potencijal u borbi protiv MRSA, uglavnom stvaranjem organskih kiselina koje ometaju metabolizam bakterija. Na osnovu rezultata našeg preglednog rada, najefikasniji bakteriocini protiv *S. aureus* su bili Enterocin AS-48 sa fenolnim jedinjenjima ili sa 2NPOH, Bacteriocin izolovan iz *Lactobacillus pentosus* - Pentocin JL-1 i bakteriocin proizveden od *S. pasteuri* RSP-1. Probiotici mogu da deluju na patogen tako što proizvode antimikrobna jedinjenja koja ometaju proizvodnju MRSA ćelija i na kraju dovode do smrti bakterija. U našem pregledom radu, najveću efikasnost pokazali su probiotici *Streptomices griseus*, *Pediococcus acidilacti* sojevi A11 i C12, *Lactococcus lactis* i *Lactobionic acid*. Pristup koji kombinuje ove prirodne agense pokazao je zadovoljavajuće rezultate u prepoznavanju i eliminaciji MRSA ćelija. Koristeći snagu prirode, razvijamo potencijal za prevazilaženje infekcija uzrokovanih sa MRSA-ma i drugim bakterijama koje su otporne na antibiotike.

Ključne reči: *Staphilococcus aureus* otporan na meticilin (MRSA), eterična ulja, bakteriofag, bakteriocin, probiotik

INTRODUCTION

Staphylococcus aureus is a gram-positive pathogenic bacterium. The ability of *S. aureus* to adhere to specific host substrates and evade host defenses (Eom, et al. 2014; Lu, et al. 2021), as well as its ability to survive in various environmental conditions while posing different virulence factors (de Oliveira, et al. 2010; Eom, et al. 2014; Burris, et al. 2015; Lu, et al. 2021), makes it highly virulent and capable of causing life-threatening infections in both humans and animals (Zhu, et al. 2015; Catteau, et al. 2017; Lalouckova, et al. 2021). Foodborne diseases caused by *S. aureus* (Lee, et al. 2009; de Oliveira, et al. 2010; Keyvan and Tutun 2019; Prastiyanto, et al. 2020; Lalouckova, et al. 2021) are generally limited to food poisoning and gastroenteritis, resulting from enterotoxins produced by *S. aureus* (Lee, et al. 2009; Zhu, et al. 2015; AL-Saadi 2016; Prastiyanto, et al. 2020).

Antibiotic resistance is one of the most significant health challenges of the

century (Lee, et al. 2009; Chang, et al. 2017; Chang, et al. 2017; Prastiyanto, et al. 2020). Antibiotic-resistant forms of *S. aureus*, such as methicillin-resistant *Staphylococcus aureus (MRSA)*, are multi-drug-resistant (Eom, et al. 2014; AL-Saadi 2016; Lu, et al. 2021) to β-lactam antibiotics (Eom, et al. 2014; Redwan et al. 2016; Catteau, et al. 2017; Lestari, et al. 2019; Prastiyanto, et al. 2020). Foodborne *MRSA* is a major concern for public health worldwide (Lee, et al. 2013; Redwan, et al. 2016; Kang, et al. 2020; Lu, et al. 2021), because it can enter the food chain as animal based food (Vaiyapuri, et al. 2019; Kang, et al. 2020) or by colonizing in food handlers and transferring from them to food (Eom, et al. 2014). A high rate of morbidity and mortality by *MRSA* have been reported worldwide (Zhu, et al. 2015; Redwan, et al. 2016; Zouhir, et al. 2016; Salem 2017; Zihadi, et al. 2019). *MRSA* has already been isolated from food, indicating that it is present as a contaminant in the food production chain (Ansari, et al. 2020; Afshari, et al. 2022). The presence of *MRSA* has been reported mainly in meat such as pork, beef, lamb, chicken, rabbit, and turkey, as well as in dairy products such as milk and cheese (Mohammed-Ali, et al 2015). This means that the food production chain is a pathway of transmission between resistant microorganisms and humans (Mohammed-Ali, et al 2015). Food safety is an important global concern in the food industry and public health. Many preservatives that are used to control microbial growth in foods not only increase the shelf-life of food products, but they also reduce the incidence of foodborne diseases (Xu, et al. 2016; Chang, et al. 2017). Due to consumer worries regarding safety of chemical preservatives utilized in food, there is an increasing need for natural alternatives that can function as food preservatives. (Gyawali, et al 2014). Therefore, it is critical to use natural agents that control or prevent foodborne pathogens, including *MRSA*, in food (Kang, et al. 2020). By utilizing these natural antimicrobials as food preservatives, the need for excessive physical and chemical food processing can be reduced while ensuring microbial safety and environmental preservation (Yusuf, 2018).

Several natural compounds from plants, animals, and microorganisms have been studied and applied in order to inhibit or control the growth of foodborne microorganisms, including *MRSA*. Plant-derived essential oils are commonly used as flavoring and preservation agents in food and drinks have antimicrobial and antioxidative activity (Cui, et al. 2018; Yuan, et al 2018).

Bacteriophages are viruses that infect bacteria and can exhibit inhibitory activity against *S. aureus*, particularly *MRSA*. Furthermore, since gram-positive bacteria lack an outer membrane, bacteriophages can directly lyse the cell wall from the outside (Lysis from without) (Lu, et al. 2021). Bacteriocins are proteins that exhibit bactericidal effects on a variety of bacteria, including *S. aureus*. They are considered as alternatives to traditional antibiotics (Zhu, et al.

2015; Chauhan, et al. 2017; Lestari, et al. 2019) and an effective approach for use in food against *MRSA* (Arumugam, et al. 2019). Probiotics are living organisms used as food additives to help maintain a healthy microbial balance in the gastrointestinal tract, leading to better health in humans (Lee, et al. 2021).

This review focuses on the effective natural antimicrobials originating from plants and microorganisms against *MRSA*, including essential oils, bacteriophages, bacteriocins, and probiotics. The mechanisms of action, as well as their effectiveness, are also surveyed. Our main aim was to review the efficiency of natural antimicrobial agents in combating *MRSA* in food.

MATERIAL AND METHODS

Study Design

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines were applied to conduct this systematic review. The main objective of this study was to review the literature on natural approaches for controlling *MRSA* in food.

Search Strategy

In this study, the main databases, including Scopus, PubMed, Google Scholar, and Science Direct, were searched. The literature review was limited to studies published from 2000 to 2023. The search was independently conducted for each database, focusing on controlling *Methicillin-resistant Staphylococcus aureus* OR *MRSA* in any food product worldwide. The keywords used were "*Methicillin-resistant Staphylococcus aureus*" OR "*MRSA*" AND "Dairy" OR "Milk" OR "Meat" OR "Food" AND "Essential Oils" OR "Probiotic" OR "Bacteriophage" OR "Bacteriocin" AND "Control".

Inclusion and Exclusion Criteria

This review included articles (n = 83) that reported on the natural types of effective antimicrobials, including essential oils, bacteriophages, bacteriocins, and probiotics, against *MRSA*. The selection for inclusion eligibility was conducted by scanning the titles, abstracts, and full texts of retrieved articles. The focus of our study was on livestock-associated *(LA) MRSA*. All review studies, duplicate publications, as well as clinical reports and trials on healthcare-associated *(HA) MRSA*, were excluded.

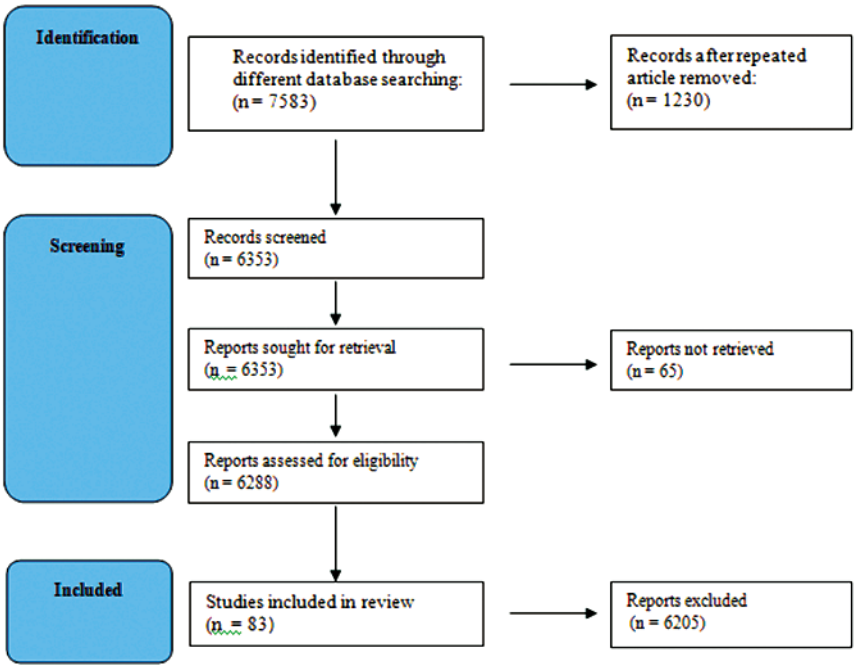

Figure 1. PRISMA flowchart for studies selection

RESULTS

Essential Oils

Essential oils have shown an antimicrobial effect against *S. aureus* and *MRSA* in particular. For instance, Cinnamon oil, Thyme oil, and Lemongrass oil reduced the *MRSA* population in minced meat by 7.6, 6.53, and 5.94 log CFU/g, respectively, when applied at a concentration of 1.5% (Eom, et al. 2014). Cinnamon oil was bactericidal against the biofilm activity of *MRSA*. A concentration of 1.0 mg/mL of cinnamon oil was sufficient to eliminate *MRSA* biofilm (Cui, et al. 2016). *Syzygium aromaticum (CLV)* and *Cinnamomum zeylanicum (CIN)* exhibited bactericidal activity at a concentration of 200 µg/mL against *S. aureus* and reduced the population of *S. aureus* by 4.50 log10 CFU/mL and 3.97 log10 CFU/mL, respectively (Mandal, et al. 2011).*Cuminum cyminum (CMN)* exhibited bactericidal activity at 300 µg/mL and caused a reduction of 0.59 log10 CFU/mL in *MRSA* after 24 hrs (Mandal, et al. 2011). The MIC concentration of the polyphenolic components of green tea, neem leaves extract, and a combination of green tea and neem were 15.62, 31.25,

and 46.87 mg/mL, respectively (Zihadi, et al. 2019). Allicin liquid was active against *S. aureus* strains, and all *MRSA* strains were inhibited by *allicin* at 32 µg/mL (Cutler, et al 2004). The indigenous cinnamon B leaf oil *(Cinnamomum osmophloeum)* had antibacterial effect against *MRSA* with an MIC of 250 µg/mL (Chang, et al. 2001). The *ethanolic* extract of *Elettaria cardamomum* displayed antibacterial activity against *MRSA*, with a minimum inhibitory concentration (MIC) of 0.25 mg/disk and minimum bactericidal concentration (MBC) of 0.50 mg/disk against *S. aureus* (Yassin, et al. 2022). *Nigella sativa* oil extract was effective against *MRSA*, with inhibition zones of 7 ± 1 mm and 10 ± 0.9 mm observed at concentrations of 400 µl and 800 µl, respectively (Abdullah, et al. 2021). *Litsea cubeba* essential oil *(LC-EO)* contained high percentages of *aldehydes*, *primarily β-Citral* (39.25%) and *α-Citral* (30.89%). *LC-EO* caused a steady decrease in *MRSA* populations, with a 99.99% reduction observed after 2 hours of treatment with 0.25 mg/mL of *LC-EO* (Hu, et al. 2019). *Red propolis* extracts *(RPE)* had an inhibition zone of 16.5 ± 0.5 mm and 19.3 ± 0.5 mm against *S. aureus* and *MRSA*, respectively (Zhang, et al. 2022). The essential oil extracted from *Carum carvi L.* seeds completely prevented *MRSA* biofilm formation at a concentration of 1.28%, with decreasing inhibitory effects observed at lower concentrations (Liu, et al. 2023). The *ethanol* extract from *Psoralea corylifolia* seeds exhibited antibacterial activity against Gram-positive bacteria, with inhibition zones of 14 mm and 16 mm for *S. aureus* and *MRSA,* respectively. *MRSA* cells treated with 1600 µg/mL of the extract were deformed and collapsed (Li, et al. 2019). *Backhousia citriodora* essential oil significantly inhibited 90.01% to 93.39% of *S. aureus* biofilms (Lim, et al. 2022). Oregano *(Origanum vulgare)* and clove *(Eugenia caryophyllata)* essential oils were effective against *S. aureus* and *MRSA*, with complete inhibition at concentrations of 0.63 µg/mL and 10 µg/mL, respectively (Debiagi, et al. 2020). Finally, *Lippia micromera* and *Plectranthus amboinicus* exhibited potent antibacterial activity against *MRSA*, with large inhibition zones of 23.7 - 35.7 mm (Bugayong, et al. 2019). The MIC of a combination of *oregano* and *thyme* essential oils was found to be 320 µg/mL (Boskovic et al. 2015) .Other studies reporting the effectiveness of essential oils are summarized in Table 1. According to Table 1, the most effective compound against *MRSA* is the *liposome* containing cinnamon oil, with a MIC of 0.25 mg/mL and MBC of 0.25 mg/mL, this compound resulted in a 99.99% decrease in *MRSA* populations after 4 hours and a decrease of 2.83 logs after 24 hours at a concentration of 1.0 mg/mL. Other most effective EOs against *MRSA* are *Cinnamomum zeylanicum*, *Syzygium aromaticum*, *Cuminum cyminum*, respectively. Additionally, *allicin*, *glabrol*, *clove buds*, and *Backhousia citriodora* essential oil s < 0, have shown significant effectiveness against *MRSA* with low MIC values.

Table 1. Effectiveness of essential oils against *MRSA*

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Cinnamon oil, Thyme oil and Lemongrass oil	*Cinnamaldehyde, eugenol, Alpha* and *beta-citral, mycrene*	The initial count of *MRSA* after inoculation (at zero time) was 10.28(log CFU/g) which at a concentration of 1.5% Cinnamon oil, Thyme oil and Lemongrass oil reduced *MRSA* population by 7.6, 6.53, 5.94 log CFU/g, respectively.	(Salem 2017)
Liposome containing cinnamon oil	*Cinnamon oil*	After 4h there was a decrease of around 99.99% in the *MRSA* populations and after 24 h, the population of *MRSA* decreased by 2.83 logs using 1.0 mg mL^{-1}. MIC (mg/mL): 0.25, MBC (mg/mL): 0.25	(Cui, et al. 2016)
Indian Spices	*Syzygium aromaticum (CLV), Cinnamomum zeylanicum (CIN) and Cuminum cyminum (CMN)*	After 24 h the *CIN* and *CLV* showed bactericidal activity at concentration 200 μg/mL against *S. aureus* reducing 4.50 log10 cfu/mL and 3.97 log10 cfu/mL, respectively; *CMN* exhibited bactericidal effects at 300 μg/mL, leaving 0.59 log10 cfu/mL. Effectiveness order: *C.zeylanicum* > *S.aromaticum* > *C. cyminum*	(Mandal, et al. 2011)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Polyphenolic components of *Green tea,* Neem leaves extract of *Camellia sinensis* and *Azadirachta indica leaves*	*Catechin*	MIC (mg/mL): *Green tea*: 15.62 *Neem*: 31.25 *Green tea + Neem*: 46.87 (*green tea* extract is more potent than *neem* against *MRSA*)	(Zihadi, et al. 2019)
Allicin	NR	MIC: 32 µg/Ml, MBC: 128 µg/mL	(Cutler, et al 2004)
Cinnamomum osmophloeum leaf	*Cinnamaldehyde*	MIC: 250 µg/mL	(Chang, et al. 2001)
Thyme (Tymus vulgaris) and Oregano (Origanum vulgare)	*Thymol* from *Thyme and Carvacrol* from *Oregano*	MIC of *Oregano* and *Thyme*: 320 µg/mL MBC of *Oregano*: 1280 µg/mL MBC of *Thyme*: 640 µg/mL	(Boskovic, et al. 2015)
PFF (phlorofucofuroecko, a marine-derived polyphenol found in brown algae)	NR	MIC: 64 µg/mL	(Eom, et al. 2014)
URS (ursolic acid 3-O-α-L arabinopyranoside was isolated from the leaves of A. henryi (Oliv) with oxacillin	Ursolic acid 3-O-α-L-arabinopyranoside with (URS) oxacillin	MIC: 6.25 µg/mL After 24 h, treatment with 1/2 MIC OXA and 3/4 MIC URS in combination resulted in combined group bacteria counts that decreased to 3 log10.	(Zhou, et al. 2017)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Pink oyster mushroom Pleurotus flabellatus	The terpenoid compound group	MIC: 62.5 mg/mL MBC: 250 mg/mL	(Ghosh, et al. 2016)
Carvacrol	NR	MIC: 0.11 mg/mL	(Keyvan, and Tutun 2019)
Bulb Eleutherine Americana	Naphtho-quinone	MIC: 125-500 g/mL, MBC: 250-1000 g/mL	(Ifesan, et al. 2009)
Aloysia citriodora essential oils from Baqa al-Gharbiyye and Umm al-Fahm	lipophilic structures like α- citral and α-curcumene	MIC: 2.5 µg/mL	(Arumugam, et al. 2019)
Garlic	Allicin (allyl 2-propenethiosulphinate)	MIC: 256 g/mL	(Prastiyanto, et al. 2020)
Cinnamon (Cinnamomum verum)	Cinnamaldehyde, eugenol	Against MSSA: MIC of 250 µg/mL Against MRSA: MIC of 250 µg/mL	(Prastiyanto, et al. 2020)
Thyme (Thymus vulgaris L.)	Thymol, carvacrol	MIC of 0.25% (v/v)	(Prastiyanto, et al. 2020)
Clove (Eugenia caryophyllata)	Eugenol, Cariofilene	MIC of 0.25% (v/v)	(Prastiyanto, et al. 2020)
Rosemary (Rosemarinus officinalis)	Borneol, 1, 8-cineole	MIC of 1.0% (v/v) MIC of 0.5% (v/v)	(Prastiyanto, et al. 2020)
Sage (Salvia officinalis)	Thujone, cineol, thymol	MIC of 1.0 (% v/v)	(Prastiyanto, A et al. 2020)
Tea tree (Melaleuca alternifolia)	Terpene	MIC of 0.5% (v/v)	(Prastiyanto, et al. 2020)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Flavonoids from licorice	glabrol, licochalcone A, licochalcone C, and licochalcone E	After 3 h, at 8 mg/mL killed both MRSA T144 and MSSA ATCC29213 completely and after 1 h, all MRSA T144 and MSSA ATCC29213 cells were killed after exposure to glabrol at 4–16 mg/mL.	(Burgos, et al. 2015)
Clove buds	Eugenol	MIC: 0.62 mg/mL	(Xu, et al. 2016)
Chuzhou chrysanthemum	B-Eudesmene, L-Borneol, Camphor	MIC: 5 mg/mL, MBC: 10 mg/mL	(Cui, et al. 2018)
Syzygium antisepticum plant	b-caryophyllene	MIC: 0.12 mg/mL MBC: 0.5 mg/mL	(Yuan, et al 2018)
Sanguisorba officinalis strains	Ethanol	At the concentration of 10 mg/mL S. officinalis the growth of the MRSA was inhibited. at a low concentration (<2.5 mg/mL), inhibitory effect of S. officinalis on biofilm formation in the MRSA strain was obvious.	(Chen, et al. 2015)
Korean soybean fermented product doenjang	Methanolic	MIC: 2048 μg/mL	(Lalouckova, et al. 2021)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Bisdemethoxycurcumin with three antibiotics (gentamicin, ampicillin and oxacillin)	NR	MIC: 7/8 µg /mL for all S. aureus strains including MRSA. The combination of BDMC with antibiotics caused more than 3 log10 cfu/mL reductions on all the three S. aureus strains.	(Hermawati, et al. 2016)
Thymol and carvacrol with organic acids (lactic acid)	NR	Combination of thymol and carvacrol with organic acids results a reduction over two log cycles in initial bacterial after 24 h.Thymol and carvacrol showed MIC of 0.6 and 1.25 µL/mL and MIC of lactic acid was 2.5 µL/mL	(de Oliveira, et al. 2010)
Elettaria cardamomum ethanolic extract	a-terpinyl acetate and 1,8 cineole	MIC: 0.25 mg/disk, MBC: 0.50 mg/disk	(Yassin, et al. 2022)
Nigella sativa (Black seed) Oil	Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL and Pentane, 2-cyclopropyl	MIC shows that at the concentration of 400 µl with (7± 1) mm of inhibition zone and 800mL concentration was (10± 0.9) mm	(Abdullah, et al. 2021)
Litsea cubeba essential oil	β-Citral and α-Citral	MIC 0.5 mg/ mL, MBC 1.0 mg/ mL	(Hu, et al. 2019)
Red Propolis	Pinobanksin, pinobanksin-3-acetate	MIC: of 50 µg/mL MBC: 200 µg/mL	(Zhang, et al. 2022)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Essential Oil Extracted from Carum carvi L. seeds (CEO)	Carvone and limonene	MIC: 6.4 µg/mL	(Liu, et al. 2023)
Ethanol Extracts of Psoralea corylifolia Seeds	Phenol, hydrazine, aldehyde, and ketone	MIC: 50 µg/mL MBC: 100 µg/mL	(Li, et al. 2019)
Black seed (Nigella sativa) oil	Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL and Pentane, 2-cyclopropyl	MIC: 32.8 mg/mL MBC 42.2 mg/mL	(Abdullah, et al. 2021)
Backhousia citriodora Essential Oil (BCEO) leaves	oxygenated monoterpenes and neral phytocompounds	MIC: 6.25 µL/mL, MBC: 50 µL/mL	(Lim, et al. 2022)
Pelargonium graveolens Oil	citronellol, citronellyl formate	MIC: 1.56 µg/mL.	(Jaradat, et al. 2022)
Origanum-vulgare and Eugenia caryophyllata oil	phenols components	MIC CEO: 10 µg/mL MIC OEO: 0.63 µg/mL	(Debiagi, et al. 2020)
Essential Oils from Leaves of Some Aromatic Plants	Monoterpenes	MIC :2.00 %, MBC>4.00 %	(Bugayong, et al. 2019)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Essential Oils from Elettaria Cardamomum fruit capsules	monoterpenes and sesquiterpenes	MIC: 250 μg/mL.	(Jha, et al. 2022)

NR: not reported

Bacteriophage

A phage endolysin, *LysP108*, was able to decrease viable *MRSA* cells by approximately 2 log units within 30 minutes at an optimal concentration of 250 μg/mL. At an MIC of 100 μg/mL, while the antibiofilm activity of the endolysin resulted in the removal of 66% of *MRSA* biofilm (Lu, et al. 2021). Endolysin *LysSA11*, at a concentration of 450 nM, reduced the optical density of the *S. aureus* culture after 30 minutes. However, the efficacy of *LysSA11* declined by 50% at temperatures of 4 °C or 65 °C (Chang, et al. 2017). Two other endolysins, *CHAPLysGH15* and *LysGH15*, that were isolated from *S. aureus*, showed a rapid antibacterial effect on *MSSA* and *MRSA* strains. Although they became inactive when exposed to heat treatment, *CHAPLysGH15* demonstrated high activity at pH 7.0–10.0, and *LysGH15* was active in high-salt environments. Therefore, they can be used in salty foods, as well as alkaline foods, including raw beef, pork, fish, and chicken, which are prone to contamination with *S. aureus* (Yan, et al. 2021). A well-studied, *S. aureus*-specific bacteriophage, Phage K, demonstrated a good inhibitory effect on *S. aureus* strains, including *MRSA*. Furthermore, when this phage was combined with essential oils, such as *a-pinene*, the inhibitory effect was greater than either the phage or the essential oil alone (Ghosh, et al. 2016). When Phage *SapYZU11* was applied at a multiplicity of infection (MOI) of 100, it resulted in the maximum reduction of *MRSA JCSC 4744* and *S. aureus* cocktail after 4 days, with reductions of 0.33 log CFU/mL and 0.29 log CFU/mL, respectively. These findings suggest that *SapYZU11* could be utilized as a biocontrol agent to effectively combat *S. aureus* contamination in the food industry (Wen, et al. 2023). Good results have been reported for combinations of phages and other antimicrobials, such as bacteriocins. For example, a combination of phage *SAP84* and a bacteriocin from *L. lactis CJNU* demonstrated significantly better *anti-S. aureus* activity

compared to each one alone *(Kim, et al. 2019)*. The synergistic inhibition of the combination of phage *SAP84* and bacteriocin against *S. aureus* caused a reduction of more than 5 log in viable counts, while the phage alone led to only about a 2 log cfu/mL reduction in *S. aureus* counts (Kim, et al. 2019). In another study, a lower concentration of endolysin *LysH5* was required in combination with subinhibitory concentrations of nisin to achieve complete inhibition of *S. aureus Sa9* (Arumugam, et al. 2019). After the treatment with 1 μM of recombinant *SAP8* endolysin, the initial *MRSA* count of 5.93 log CFU/mL was reduced to 3.64 log CFU/mL. In addition, the combination of 0.01 μM of recombinant *SAP8* endolysin and 18 IU/mL of nisin completely prevented the growth of *MRSA* (Hassan, et al. 2020). Also, the combination of bacteriophage endolysin *LysSA97* with *carvacrol* was found to significantly decrease the number of viable *S. aureus* cells (Chang, et al. 2017). When a combination of *S. aureus* phage (MOI 10) and 1% thyme oil was used, a greater reduction (87.22%) in *S. aureus* was achieved compared to using each treatment alone (Abdallah, et al. 2021). These examples indicate that phages can have a synergistic effect with other antibacterials. The effects of different bacteriophages and endolysins against *S. aureus*, including *MRSA*, have been reported in studies that are summarized in Table 2. Based on Table 2, the most effective phage compounds are *S. aureus* phage (containing *CHAPLysGH15* and *LysGH15)*, phage *SA11* endolysin *LysSA11*, endolysin *LysSA97* with *carvacrol*, and phage *endolysin LysH5* and *nisin*, respectively.

Table 2. Effect of bacteriophages and endolysins against *MRSA*

Bacteriophage			
Name	**Active Components**	**Effectiveness**	**References**
Endolysin *LysP108*	NR	MIC: 100 μg /mL	(Lu, et al. 2021)
Staphylococcus aureus bacteriophage	*CHAPLysGH15 and LysGH15*	*MRSA* was completely cleaved by 0.4 nmol/cm2 of *CHAPLysGH15*. 1.0 Log10 cfu/cm2 of *MRSA* declined after adding 0.4 nmol/cm2 of *LysGH15*	(Li, et al. 2011)

Bacteriophage			
Name	**Active Components**	**Effectiveness**	**References**
Endolysin *LysSA97* (an endolysin encoded by the bacteriophage *SA97*) with *carvacrol*	NR	The numbers of *S. aureus cells* were decreased by 0.8 ± 0.2 log cfu/mL and 1.0 ± 0.0 log cfu/mL at concentrations of 376 nm and 3.33 mm, respectively.	(Chang, et al. 2017)
EOCs (*a-pinene and 3-carene*) combined with two types of *S. aureus* bacteriophage, *phage K (ATCC 19685-B1)* and *phage 92 (ATCC 33741-B1)*	NR	Both phage of *S. aureus*-specific bacteriophage alone and EO *(a-pinene)* alone at 1.5 and 3.28 % yielded similar inhibition trends. However, with *phage K* and EOC (essential oil compounds) combinations, *phage K* with 3.28 % *a-pinene* inhibited *S. aureus* growth better than other combinations of EOCs and phage depending on the strain.	(Jaradat, et al. 2021)
Phage SA11 endolysin *LysSA11*	NR	The highest dose of Phage *SA11 endolysin LysSA11*(450 nM of endolysin) yielded a 50% reduction in optical density in less than 20 min and a 70% reduction within 30 min. *LysSA11* treatment (3.37μM, 1 h) reduced the number of *staphylococcal cells* in milk by about 2.53 log/mL	(Chang, et al. 2017)
Phage endolysin *LysH5* and *nisin*	NR	The MICs of *nisin* and *LysH5* were 3μg/mL and 50u/mL, respectively but in the presence of subinhibitory concentrations of *nisin*, a lower endolysin concentration was needed to fully inhibit *S. aureus Sa9*. These values implied up to a 64-fold and 16-fold reduction of the *nisin* and endolysin MICs, respectively, when used in combination.	(Arumugam, et al. 2019)

NR: not reported

Bacteriocins

According to studies, the growth of gram-positive pathogens, including *S. epidermidis*, *S. aureus*, and *MRSA*, was effectively inhibited by *NX371*, a novel class III bacteriocin gene. When *NX371* was added to milk, it moderately but significantly inhibited the growth of pathogens from day 1 to day 7, with reductions of 3.5 - 4.0 log in milk and 5.0 - 7.0 log in cheese, indicating its effectiveness as a food additive for controlling *S. aureus* in dairy products (Meng, et al. 2021). *Colicin* and *interocin* bacteriocins produced by *Escherichia coli* strains and *Enterococcus* species were found to have bactericidal effect against *MRSAs* and other *Staphylococcal* isolates, with complete bactericidal action achieved after 18-24 hours of incubation (Bajlan, et al. 2018). Bacteriocin produced by *Lactobacillus plantarum ZJ217 (plantaricin ZJ217)* was found to significantly decrease the colony forming units (Log10 CFU) of *S. aureus*, with viable cell counts decreasing from 6.5 ± 0.1 to 3.7 ± 0.04 log CFU/mL within 2 hours of incubation (Zhu, et al. 2015). Bacteriocin *KTH0-1S* produced by *Lactococcus lactis KTH0-1S* was found to significantly reduce the viable cell counts of *S. aureus* within 2 hours of incubation, with a higher proportion of dead cells compared to the control treatment (Saelao, et al. 2017). Bacteriocin *Paracin 54* produced by *Lactobacillus paracasei ZFM54*, was found to have a strong inhibitory effect on *Staphylococci*, with minimum inhibitory concentration values of 3.00 - 4.50 μg/mL (Zhu, et al. 2021). Bacteriocin producing *Pseudomonas aeruginosa TA6*, isolated from soil, was found to decrease the cell density of *S. aureus* rapidly, with cell lysis eventually occurring at concentrations of 100 AU/mL (Arumugam, et al. 2019). *Plantaricin 827*, produced by *Lactobacillus plantarum 163*, was found to quickly decrease *S. aureus* cells within 150 minutes of treatment with 64 μg/mL, and all *S. aureus* cells were destroyed within 90 minutes of treatment with 128 μg/mL. Moreover, *plantaricin 827* exhibited a certain preservation effect in skimmed milk and significantly extended the shelf life of skimmed milk (Zhao, et al. 2022). Bacteriocins produced by two strains, *Lactobacillus helveticus (BLh)* and *Lactobacillus plantarum (BLp*), had significant activity against *S. aureus* and *MRSA*. *L. helveticus (BLh)* was the most effective against *MRSA* after 18 to 24 hours of incubation at 37°C, while *L. plantarum (BLp)* had a similar effect against *MRSA* after 24 to 48 hours of incubation at 37°C under anaerobic conditions. The bacteriocin extracted from *L. plantarum (BLp)* was active even after passing through high temperature and pressure during sterilization, but the bacteriocin synthesized by *L. helveticus (BLh)* was more labile to heat (Hassan, et al. 2020). *Nisin*, a bacteriocin produced by the *Lactococcus lactis subsp. lactis* bacterium, exhibited

bacteriostatic activity against *MRSA* alone and had no effect against *S. aureus ATCC 25937*, while some strains of *Lactobacillus reuteri* produced *reuterin (β-hydroxypropionaldehyde)* under anaerobic conditions, which was considered to have bactericidal effects against *MRSA* and *S. aureus ATCC25937*. The combination of *nisin* at a concentration of 25.6 and *reuterin* at a concentration of 5.2 mg/mL exerted a bactericidal effect on *MRSA* and *S. aureus ATCC 25937* (Yehia, et al. 2022). Combinations of bacteriocins with other antimicrobials can increase their antibacterial efficacy. For instance, co-treatment of drinks with *enterocin* and phenolic compounds *(2NPOH)* resulted in the eradication of viable *staphylococci* after 24 hours (Burgos, et al. 2015). The effects of different types of bacteriocins against *S. aureus*, including *MRSA*, have been reported in studies that are summarized in Table 3. According to Table 3, the most effective bacteriocins against *S. aureus* are *Enterocin AS-48* with phenolic compounds or with *2NPOH*, Bacteriocin isolated from *Lactobacillus pentosus – Pentocin JL-1*, bacteriocin producing *Pseudomonas aeruginosa TA6*, and bacteriocin produced by *S. pasteuri RSP-1,* respectively.

Table 3. Effect of some bacteriocins against *MRSA*.

Bacteriocins			
Name	**Active Components**	**Effectiveness**	**References**
Bovine myeloid antimicrobial peptide (BMAP-28)	NR	20 mg/mL of *BMAP-28* could inhibit the growth of the two kinds of bacteria (*MRSA* and *MSSA*). MIC range (mg/mL): 5–20	(Takagi, et al. 2012)
Cell-free extracts of *Bifidobacterium*	*b1, b2, BL* and *BI*	MIC: 1.0 mg/mL	(AL-Saadi 2016)
Bacteriocin Produced by *B. cereus TSH5*	NR	MIC: 80 μg/mL	(Chauhan, et al. 2017)
Bacteriocin produced by *Staphylococcus pasteuri RSP-1 (S. pasteuri RSP-1)*	NR	MIC: 5 AU/mL	(Hong, et al. 2018)

Bacteriocins			
Name	**Active Components**	**Effectiveness**	**References**
Bacteriocin isolated from *Lactobacillus pentosus*	*Pentocin JL-1*	MIC: 7.5 µg/mL	(Jiang, et al. 2017)
Enterocin *AS-48* with phenolic compounds or with *2NPOH*	NR	No viable *staphylococci* were detected after 24 h incubation with the combination of *enterocin AS-48* and *2NPOH*	(Murray, et al. 2021)
Bacteriocin from *Lactococcus lactis KU24*	*Bacteriocin KU24*	*S. aureus ATCC 33591* was inhibited *by bacteriocin KU24* at 2 Log cfu/mL after 10 h of incubation. MIC: 400 to 800 AU/mL	(Lee, et al. 2013)
Bacteriocin producing *Pseudomonas aeruginosa TA6*	NR	MIC: 50 AU/mL the cell density of *S. aureus* decreased rapidly, and cell lysis occurred at 100 AU/mL concentrations	(Zhou, et al. 2017)
Bacteriocin producing *Lactobacillus acidophilus*	*bacteriocin gene NX371*	MIC90 was ranged from 20 to 160 µg/mL	(Meng, et al. 2021)
Bacteriocins produced by *Escherichia coli* and *Enterococcus species*	*Colicin and interocin*	The incubation times for complete bactericidal action were 18-24h.	(Bajlan, et al. 2018)
Bacteriocin produced of *Lactococcus lactis KTH0-1S*	*Bacteriocin KTH0-1S*	The proportion of dead cells was significantly higher since viable cell counts decreased from 6.5±0.1 to 3.7±0.04 log CFU/mL within 2 h of incubation	(Saelao, et al. 2017)
Bacteriocin produced of *Lactobacillus paracasei ZFM54*	*Bacteriocin Paracin 54*	MIC: 3.50 µg/mL	(Zhu, et al. 2021)

Bacteriocins			
Name	**Active Components**	**Effectiveness**	**References**
Bacteriocin producing from *Pseudomonas aeruginosa TA6*	NR	Maximum bacteriocin activity (100AU/mL) was observed at 37 °C in 24 h time duration.	(Arumugam, et al. 2019)
Bacteriocin produced by *Lactobacillus plantarum 163*	*Plantaricin 827*	MIC: 64 μg/mL.	(Zhao, et al. 2022)
Bacteriocin produced by Lactobacillus helveticus and Lactobacillus plantarum		L. helveticus showed the activity against MRSA after 18 to 24 hours of incubation at 37°C. In comparison, L. plantarum showed similar activity against MRSA after 24 to 48 hours of incubation at 37°C under anaerobic conditions.	(Hassan, et al. 2020)
Bacteriocin produced by Lactococcus lactis subsp. lactis. and Lactobacillus reuteri	*Nisin and reuterin*	MIC of nisin: 51.2 mg/ mL, MIC of reuterin: 5.2mg/mL MBC of nisin: 5 mg/mL, MBC for reuterin: 5 mg/mL	(Yehia, et al. 2022)

NR: not reported

Probiotics

The most common probiotics are *lactic acid bacteria (LAB)* strains, and they are considered safe. *LAB* can produce bactericidal bioactive peptides and enzymes that have antibacterial and antibiofilm effects (Hermawati, et al. 2016). For instance, *Lactobacillus* can inhibit *Staphylococcal cells*, including *MRSA* (Hermawati, et al. 2016). Several probiotics such as *Lactobacillus plantarum* (Lee, et al. 2021, Afshari, et al. 2022), *Lactobacillus acidophilus*, *Lactobacillus casei* (Hermawati, et al. 2016), *Streptomyces griseus*, *Lactococcus lactis*, *Streptococcus*, *Leuconostoc*, and *Pediococcus* (Li, et al. 2011) have demonstrated

inhibitory effects on *S. aureus* strains, including *MRSA*. Table 4 shows the effects of different probiotics on *MRSA*. According to Table 4, the most effective probiotics were *Streptomyces griseus*, *Pediococcus acidilactici strains A11 and C12, Lactococcus lactis*, and *Lactobionic acid*, respectively.

Table 4. Effect of some probiotics against MRSA

Probiotic			
Name	**Active Components**	**Effectiveness**	**References**
Lactobacillus acidophilus and probiotic *Lactobacillus casei*	NR	MIC: 3.12% for *Lactobacillus acidophilus* and 2% *Lactobacillus casei*	(Karska-Wysocki, et al. 2010)
Probiotic *Lactobacillus plantarum KU200656*	NR	MIC :12.5%	(Lee, et al. 2021)
Pseudomonas fluorescens	*Pseudomonic acids and Mupirocin*	MIC of 8-256 µg/mL for low level resistance and 512 µg/mL for high level resistance	(Prastiyanto, et al. 2020)
Streptomyces griseus	*Treptose, streptydine, and Nmethyl- L –glycosamine and Streptomycin*	MIC: 1.56-6.25 µg/mL	(Prastiyanto, et al. 2020)
Lactococcus lactis	*Lnathionine (Lan), methyllanthionine (MeLan), didehydroalanine (Dha) and didehydroaminobutyric acid (Dhb) and Nisin*	MIC: 1.5 to > 1.6 mg/L	(Prastiyanto, et al. 2020)
Streptococcus, Leuconostoc, Lactobacillus, and *Pediococcus*	*Diacetyl*	MIC: 1.00 µg/mL	(Prastiyanto, et al. 2020)
Pediococcus acidilactici strains A11 and C12	NR	MIC: 25%, MBC: 12.5%	(Lestari, et al. 2019)

NR: not reported

DISCUSSION

Essential oils

Several possible mechanisms have been proposed for antibacterial activity of essential oils. Table 4 shows the mechanisms of action of reported EOs. Essential oils can inhibit enzymes and compounds that are needed for the growth of *MRSA* (Eom, et al. 2014; Burris, et al. 2015). For example, *cinnamaldehyde* in cinnamon oil inhibits *N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL)* and *AI-2* (Salem 2017) while allicin liquid can inhibit sulfhydryl enzymes, thereby inhibiting DNA and protein synthesis (Cutler, et al 2004; Li, et al. 2011). Some EOs can cause the release of intracellular components (Eom, et al. 2014; Jaradat, et al. 2021), such as brown algae, which lead to the release of intracellular components via phlorofucofuroeckol (PFF) (Eom, et al. 2014). Inhibition of enzymes by EOs can occur through participation in electron transport with the cell components and binding to bacterial adhesions and cell walls (Ifesan, et al. 2009). *Aloysia citriodora* EO can participate in the lipophilic lipids of the mitochondria and cytoplasmic membrane due to their lipophilic ability (Jaradat, et al. 2021). The lipophilic characteristics of EOs make them capable of easily penetrating the bacterial cell (Prastiyanto, et al. 2020). For example, *terpenoids* in mushroom *Pleurotus flabellatus* have this ability and can interfere with protein synthesis and DNA replication (Prastiyanto, et al. 2020). Additionally, some EOs such as EOs derived from *Chuzhou chrysanthemum* and *clove buds* increase the permeability of the cell membrane, resulting in the leakage of intracellular essential substances such as electrolytes, protein, and nucleic acids (Xu, et al. 2016; Cui, et al. 2018). Many EOs such as *carvacrol* are safe to apply in foods as a natural food preservative and are 'Generally Recognized as Safe' (GRAS) by the US Food and Drug Administration (FDA) (Chang, et al. 2017). EOs can have an enhanced effect when they are used at high concentrations (Higginbotham, et al. 2014). Furthermore, if they are applied in processed foods such as hot dogs, chemicals like potassium lactate, sodium lactate, sodium diacetate, and sodium nitrite, they can improve the antimicrobial activity of the Eos (Higginbotham, et al. 2014). Black seed *(Nigella sativa*) is a type of medicinal herb that contains bioactive substances of medical importance. The GC-MS analysis of *N. sativa* shows that it contains five essential compounds, all of which are a unique mix of organic compounds and alkaloids that possess high biological activity, such as *Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL, and Pentane, 2-cyclopropyl* (Abdullah, et al. 2021). *Litsea cubeba* essential oil *(LC-EO)* can cause *MRSA* cell rupture, which results in the leakage of cellular content and ultimately

leads to the bacteria's death. LC-EO treatment decreases the activity of four ATPases, including the Na+/K+ ATPase, Ca2+/ Mg2+ATPase, Ca2+ATPase, and Mg2+ATPase (Hu, et al. 2019). *Chinese Red Propolis* is rich in *pinobanksin* and *pinobanksin-3-acetate*, and its antibacterial activity may be the result of the synergistic effect of polyphenols (Zhang, et al. 2022). *Carum carvi L.* disrupts *MRSA* biofilm and amino acid metabolism, and it also hinders DNA and RNA synthesis (Liu, et al. 2023). *Psoralea corylifolia* seed ethanol extract *(PCEE)* is composed of *phenol*, *hydrazine*, *aldehyde*, and *ketone*, which can destroy the cell structure and reduce enzymes, ultimately killing bacteria (Li, et al. 2019). *Backhousia citriodora* Essential Oil *(BCEO)* contains large amounts of *oxygenated monoterpenes*, which disrupt the microbial cytoplasmic wall, improve cell permeability, and lead to cell death (Lim, et al. 2022). Oregano essential oil (OEO) and cinnamon essential oil (CEO) increase cell permeability and cause leakage of intracellular constituents, leading to the disruption of the cell respiration system and microbial enzyme system (Debiagi, et al. 2020). *L. micromera* and *P. amboinicus* essential oils contain *monoterpenes* such as *carvacrol*, *γ-terpinene*, and *β-cymene*, which are responsible for their antibacterial activity against *Staphylococcus species* including *MRSA* (Bugayong, et al. 2019). *Elettaria cardamomum* essential oil blurs the surface barrier of the cell wall, altering the structure of the cells, and causing bacterial mortality (Jha, et al. 2022). The combination of EOs can enhance the efficacy of their antibacterial activity. For instance, when *carvacrol* and *thymol* are combined with organic acids, a significant reduction in the number of *S. aureus* is observed on food samples. On one hand, these EOs disrupt the bacterial cell membrane and make the bacteria more susceptible to the acidic environment. On the other hand, organic acids enhance the hydrophobicity of EOs and make the EOs bind better to hydrophobic regions of the membrane proteins (de Oliveira, et al. 2010). Thyme oil, when combined with lytic *S. aureus* phage, is a promising biocontrol agent and antimicrobial alternative in the food industry to control and reduce *MRSA* or other *antibiotic-resistant S. aureus* contamination in food (Abdallah, et al. 2021). Some plant extracts can increase the effectiveness of antibiotics against *MRSA*. For instance, *ursolic acid 3-O-α-L-arabinopyranoside (URS)* from the leaves of *Acanthopanax henryi* (Oliv.) can enhance the *anti-MRSA* effect of oxacillin (Zhou, et al. 2017).

Bacteriophages

Bacteriophages encode peptidoglycan hydrolases, known as endolysins or lysins, which lyse bacterial cells by targeting their cell wall, particularly in Gram-positive bacteria, due to their naturally exposed peptidoglycan layer (Murray,

et al. 2021). Bacterial death by endolysins is in accordance with the typical phenomenon of osmotic-mediated cell lysis, which occurs in Gram-positive bacteria following a phage attack (Lu, et al. 2021). For instance, *LysP108* causes disintegration of the *MRSA* cell wall (Lu, et al. 2021). It has been reported that a combination of endolysin *LysSA97* with *carvacrol* can cleave bacterial peptidoglycan layers and destroy the structure of the cell wall (Chang, et al. 2017). Combining endolysins with antibiotics causes better accessibility of antibiotics to *MRSA* cells through initial lysing of the biofilm by endomysia (Linden, et al. 2015). Combining endolysins with bacteriocins can result in a higher sensitivity of *S. aureus* cells to these antibacterials. The mechanism might be attributed to the prevention of peptidoglycan breaks produced by endolysins from contraction (Arumugam, et al. 2019). Moreover, bacteriocins can cause a partial activation of autolysins that allows for better activity of the endolysin (Arumugam, et al. 2019). The combination of synthetic *SAP8 endolysin* and *nisin* can effectively restrain various types of Gram-positive bacteria by creating openings in the bacterial cell membrane and blocking the production of cell walls (Kim, et al. 2022).

Bacteriocin

Bacteriocins can cause damage to the cell wall or induce cell lysis (Lee, et al. 2013). They target the cytoplasmic membrane of bacterial cells and inhibit the proton motive force (PMF), leading to inhibition of protein or nucleic acid production (Lestari, et al. 2019). The *anti-MRSA* activity of bacteriocins is mainly due to the generation of organic acids such as *lactic acid* and *acetic acid*. These acids enter bacterial cells and interfere with essential metabolic processes (AL-Saadi 2016). Bacteriocins against *MRSA* can change the cell surface from smooth to rough. Therefore, the suggested mechanism is related to the bacterial cell membrane (Takagi, et al. 2012). Several bacteriocins have been reported to have *anti-MRSA* activity, leading to the disruption in the integrity and uniformity of *MRSA* (Zhu, et al. 2015; Jiang, et al. 201; Taggar, et al. 2021). Through bioinformatic analysis of *Lactobacillus acidophilus*, a new class III bacteriocin gene called *NX371* was discovered, which demonstrated high antimicrobial activity across a wide range of pH values (3.0-8.0). This bacteriocin was able to disrupt the cell wall of gram-positive bacteria and induce membrane leakage in gram-negative bacteria, leading to separation of the cell wall and membrane (Meng, et al. 2021). Other bacteriocins such as *colicins* and *enterocins* also exhibit antibacterial effect against Gram-positive bacteria, with *colicins* acting as transmembrane proteins that depolarize the cytoplasmic

membrane and kill cells by producing pores or acting as a nuclease to chop up DNA or RNA (Bajlan, et al. 2018). Another example is *plantaricin ZJ217*, a novel bacteriocin produced by *Lactobacillus plantarum ZJ217*, which was inhibitory effect against a variety of gram-positive and gram-negative bacteria by forming pores in cells (Zhu, et al. 2015). Similarly, bacteriocin *KTH0-1S* produced by *Lactococcus lactis KTH0-1S* acted on sensitive cells by forming pores in membranes, leading to cell death due to the loss of essential intracellular substances (Saelao, et al. 2017). *Paracin 54*, a bacteriocin produced by *Lactobacillus paracasei ZFM54*, also formed pores in the cell membrane of *MRSA*, which disrupted the balance of ions inside and outside the membrane and led to the dissipation of proton driving force, inhibiting the synthesis of intracellular ATP and causing the disorder of intracellular energy metabolism (Zhu, et al. 2021). Another bacteriocin, *plantaricin 827*, produced by *Lactobacillus plantarum 163*, had antibacterial effects against *MRSA* by increasing the cell membrane permeability and integrity, resulting in the leakage of K+ and changes in cell morphology, inhibiting biofilm formation, and interacting with genomic DNA minor groove in AT-rich regions (Zhao, et al. 2022). The combination of *nisin* produced by *Lactococcus lactis subsp. lactis* and *reuterin* produced by *Lactobacillus reuteri* also disrupted membranes by forming pores, inhibiting energy production and biosynthesis of proteins and nucleic acids (Yehia, et al. 2022). Despite the fact that they are proteins, some bacteriocins can remain stable in harsh environmental conditions. For instance, *Paracin 54* retained 93.7% of its activity after treatment with lysozyme, indicating its potential for use in food preservation. Furthermore, *Paracin 54* maintained its inhibitory activity against *MRSA* at different temperatures, suggesting its potential use in pasteurized products (Zhu, et al. 2021). *Plantaricin* 827 also exhibited antibacterial activity at pH 7.0, while *plantaricin ZJ217* was stable at pH 2.0 to 6.0 but lost activity at pH 10.0 (Zhu, et al. 2015).

Probiotics

The major effects of probiotics include modulation of the immune system, inhibition of pathogen adhesion to epithelial cells, and generation of antimicrobial compounds (AL-Saadi 2016; Lee, et al. 2021). Antimicrobial compounds produced by probiotics can also demonstrate anti-adhesion ability (Lee, et al. 2021). These antimicrobial components include organic acids, oxygen catabolites, and proteinaceous compounds (Lee, et al. 2021). These components can inhibit the growth of *MRSA* cells in food products (Karska-Wysocki, et al. 2010). For instance, *Lactococcus lactis* can generate antibacte-

rial agents, including *didehydroaminobutyric acid (Dhb)* and *didehydroalanine (Dha)*, *methyllanthionine (MeLan)*, *Lanthionine (Lan)*, and bacteriocin *(Nisin)*. These substances can disrupt the uptake of amino acids by *S. aureus* cells and suppress the production of the cell wall. In addition, some metabolites will be released, leading to cell death (Li, et al. 2011). Generally, *LBA* can cause alkaline phosphatase leakage from *MRSA cells* to the extracellular medium, and in this way, they prevent the formation of biofilms (Kang, et al. 2020). A list of *anti-S. aureus* agents and their modes of action is in Table 5.

Table 5. Mechanisms of action of anti-MRSA EOs, bacteriophages, bacteriocins, and probiotics.

Name	Active Components	Mechanisms	References
Essential oils			
Essential oils: Cinnamon oil, Thyme oil and Lemongrass oil	*Cinnamaldehyde, eugenol, Alpha and beta-citral, mycrene*	These can inhibit *N-3-oxohexanoyl- Lhomoserine lactone (3- oxo-C6-HSL)* and *AI-2,* and certain enzymes needed for the growth of *MRSA*.	(Salem 2017)
Liposome containing cinnamon oil	NR	The damage of bacterial cell membrane is by their effect on morphology, structure, function, modification in the transport of nutrients, membrane disruption, extensive leakages from the bacterial cells leading to cell death.	(Cui, et al. 2016)
Indian Spices	*Syzygium aromaticum (CLV) and Cinnamomum zeylanicum (CIN) and Cuminum cyminum (CMN)*	These can affect the synthesis of the peptidoglycan layer of the cell wall and the mode of action of the spice extracts is cell wall related.	(Mandal, et al. 2011)

Name	Active Components	Mechanisms	References
Essential oils			
Camellia sinensis and Azadirachta indica leaves	*catechin*	The *catechin* has direct effects on the destruction of the bacterial cell membrane by binding with the lipid bilayer.	(Zihadi, et al. 2019)
Allicin	NR	Inhibit the acetyl coA forming system, to inhibit DNA and protein synthesis, and to target RNA polymerase.	(Cutler, et al 2004)
Cinnamomum osmophloeum leaf essential oils	*cinnamaldehyde*	NR	(Chang, et al. 2001)
Thyme (Tymus vulgaris) and Oregano (Origanum vulgare) essential oils	*Thymol* from *Thyme* and *Carvacrol* from *Oregano*	Phosphate ion leakage.	(Boskovic, et al. 2015)
PFF (phlorofucofuroeckol, a marine-derived polyphenol found in brown algae)	NR	Interfering with cell wall synthesis and the cell membrane and agents change membrane function and permeability, leading to cell damage or death.	(Eom, et al. 2014)

Name	Active Components	Mechanisms	References
Essential oils			
URS (ursolic acid 3-O-α-L arabinopyranoside was isolated from the leaves of A. henryi (Oliv) with oxacillin	Q	Deformation of bacterial cells. Cell membrane disintegration, cell lysis and release of cytoplasmic contents.	(Yan, et al. 2021)
Pink oyster mushroom Pleurotus flabellatus	The terpenoid compound group	These penetrate the bacterial cell and may interfere with protein synthesis and DNA replication.	(Ghosh, et al. 2016)
Amomum villosum Lour	Bornyl acetate	Leakage of intracellular macromolecular substances.	(Tang, et al. 2020)
Bulb Eleutherine Americana	Naphthoquinone	Inhibits electron transport with the cell components. They also can bind to bacterial adhesions and complex with cell wall, thus inactivating enzymes.	(Ifesan, et al. 2009)
Aloysia citriodora essential oils EOs from Baqa al-Gharbiyye and Umm al-Fahm	lipophilic structures like α- citral and α-curcumene	Their lipophilic ability to partition in the lipophilic lipids of the mitochondria and cytoplasmic membrane. They could also disturb the structures, resulting in leakage of bacterial cell contents.	(Arumugam, et al. 2019)

Name	Active Components	Mechanisms	References
Essential oils			
Garlic	Allicin (allyl 2-propenethiosulphinate)	1) The primary mechanism of allicin centers on its ability to inhibit sulfhydryl enzymes common for pathogenic bacteria. 2) Inhibiting enzymes associated with DNA and protein synthesis and limiting RNA polymerase and alcohol dehydrogenase activities.	(Prastiyanto, et al. 2020)
flavonoids from licorice	glabrol, licochalcone A, licochalcone C, and licochalcone E	Disruption of membrane permeability. The binding of these to the cell wall or the cytoplasmic membrane is important for their action on the bacterial membrane.	(Burgos, et al. 2015)
Clove buds	Eugenol	The permeability of bacterial membrane would be increased, which caused the leakage of intracellular ingredient, especially losses of electrolytes including K+, Ca2+, Na+, as well as cell constituents such as protein, nucleic acids, and some essential molecules.	(Xu, et al. 2016)
Chuzhou chrysanthemum	B-Eudesmene, L-Borneol, Camphor	Disruption of the cell membrane and leakage of DNA, protein and ATP to the bulk solution.	(Cui, et al. 2018)

Name	Active Components	Mechanisms	References
Essential oils			
Syzygium antisepticum plant	b-caryophyllene	Membrane-disrupting effect was observed	(Yuan, et al 2018)
Korean soybean fermented product doenjang	Methanolic	Inhibits the respiratory metabolism and protein synthesis of the bacteria and prevents nucleic acid synthesis. Thus, it affects the integrity of the cell wall and membrane.	(Lalouckova, et al. 2021)
Bisdemethoxycurcumin with three antibiotics (gentamicin, ampicillin, and oxacillin)	NR	The polyphenol structure can destroy the cell wall of bacteria and thus increases the efficiency of antibiotics entering the cell.	(Wang, et al. 2020)
Thymol and carvacrol with organic acids (lactic acid)	NR	Phenolic compounds can damage cellular membrane changing their structure and function and causing it to become more susceptible to acid environments. On the other hand, at low pH the molecules of thymol and carvacrol are mostly dissociated, more hydrophobic, and bind better to hydrophobic regions of the membrane proteins resulting in better partition into the lipid phase of the bacterial membrane.	(de Oliveira, et al. 2010)

Name	Active Components	Mechanisms	References
Essential oils			
Litsea cubeba essential oil	β- Citral and α-Citral	The LC-EO could lead to the rupture of MRSA cells and the loss of cellular contents and eventually to the death of bacteria.	(Hu, et al. 2019)
Red Propolis	Pinobanksin, Pinobanksin-3-acetate	The mechanism of action of RPE due to loss of membrane integrity.	(Zhang, et al. 2022)
Ethanol Extracts of Psoralea corylifolia Seeds	phenol, hydrazine, aldehyde, and ketone	PCEE could change the membrane integrity of MRSA, releasing nucleic acids and proteins, resulting in bacterial death.	(Li, et al. 2019)
Manuka EO was extracted from manuka leaves	Sesquiterpenes	MIC: 0.233 mg/mL, MBC: 0.466 mg/mL	(Pedonese, et al. 2022)
Essential Oils from Elettaria Cardamomum fruit capsules	Monoterpenes and sesquiterpenes	Elettaria cardamomum EO damages the biofilm barrier, causing the bacteria to lose metabolic activity.	(Jha, et al. 2022)
Bacteriophages			
Phage Endolysin *LysP108*	NR	*LysP108* disintegrated the cell wall of *MRSA*.	(Lu, et al. 2021)

Name	Active Components	Mechanisms	References
Bacteriophages			
Bacteriophage *endolysin plygrcs*	*PlyGRCS*	The endolysin *plygrcs* would provide the initial disturbance to the biofilm structure.	(Linden, et al. 2015)
Carvacrol and lyssa97	NR	*LysSA97* cleaving bacterial peptidoglycan layers is likely to render the cell wall structure less rigid so that *carvacrol* may more readily reach the cytoplasmic membrane of S. *aureus*.	(Chang, et al. 2017)
Phage endolysin lysh5 and nisin	NR	*LysH5 activity might be increased by the permeabilization of the cytoplasmic membrane by nisin. Also, partial activation of autolysins by nisin may occur and facilitate LysH5 activity.*	(García, et al. 2010)
SAP8 endolysin	NR	*Forms pores in bacterial cytoplasmic membrane and inhibits cell wall synthesis*	(Kim, et al. 2022)
Bacteriocins:			
Bovine myeloid antimicrobial peptide (BMAP-28)	NR	1) Cell wall permeation is made by the activity of *BMAP-28* as it is diffusing inside the bacteria. 2) Bacterial smooth surface somehow changes into a rough surface by the activity of *BMAP-28*. 3) *BMAP-28* can break *MRSA* cell membranes.	(Takagi, et al. 2012)

Name	Active Components	Mechanisms	References
Bacteriocins			
Pediococcus acidilactici strains A11 and C12	NR	The initial bacteriocin reaction is to damage membrane permeability and eliminate proton motive force *(PMF)* thereby inhibiting energy production and biosynthesis of proteins or nucleic acids. 2) bacteriocin molecules are in direct contact with cell membranes, this contact process is able to disrupt membrane potential in the destabilizing cytoplasmic membranes so that cells become less strong, and membrane instability is capable of producing holes in cell membrane through the process of interference with PMF (Proton Motive Force).	(Lestari, et al. 2019)
Cell-free extract of *Bifidobacterium Species of LAB*	*b1, b2, BL* and *BI*	The acids produced by *LAB* enter the sensitive bacterial cells and interfere with the necessary metabolic process such as substrate translocation and oxidative phosphorylation, which leads to a decrease in the internal pH of bacterial cells.	(AL-Saadi 2016)

Name	Active Components	Mechanisms	References
Bacteriocins			
bacteriocin produced by *Staphylococcus pasteuri RSP-1 (S. pasteuri RSP-1)*	*Pasteuricin*	*Pasteuricin* rapidly damaged the membrane of viable cells.	(Hong, et al. 2018)
Bacteriocin isolated from *Lactobacillus pentosus*	*Pentocin JL-1*	*Pentocin JL-1* targets the cell membrane of *MRSA GIM 1.771*, causing a loss of PMF in only a few minutes, and that has a dramatic impact on the structure and integrity of the *MRSA* cell and finally leads to cell death.	(Jiang, et al. 2017)
Bacteriocin from *Lactococcus lactis KU24*	*Bacteriocin KU24*	The bacteriocin *KU24* damages the cell wall or induces cell lysis and has an impact on the bacterial cytoplasmic membrane.	(Lee, et al. 2013)
Bacteriocin from Lactobacillus plantarum ZJ217	*NR*	Bacteriocin produced by Lactobacillus plantarum can cause the formation of pores on bacterial cells and releasing ATP, and bacterial death	(Zhu, et al. 2015)
Bacteriocin isolated from the natural inhabitant of Allium cepa	*Peptide-Ba49*	Peptide-Ba49 can result in the rupturing and uniformity of MRSA.	(Taggar, et al. 2021)

Name	Active Components	Mechanisms	References
Bacteriocins			
Bacteriocin producing Lactobacillus acidophilus	*Bacteriocin gene NX371*	The leakage of intracellular ATP, disrupt the cell wall, and induce membrane leakage.	(Meng, et al. 2021)
Bacteriocin produced by Escherichia coli strains and Enterococcus species	*Colicins and Enterocin*	These depolarize the cytoplasmic membrane, leading to dissipation of cellular energy and killing domain may produce a pore in the target cell membrane, or act as a nuclease to chop up the DNA or RNA of the target cell	(Bajlan, et al. 2018)
Bacteriocin produced by Lactobacillus plantarum ZJ217	*Plantaricin ZJ217*	The Plantaricin ZJ217 had activity against biofilm cells of MRSA by forming pores to release ATP.	(Zhu, et al. 2015)
Bacteriocin produced of Lactococcus lactis KTH0-1S	*Bacteriocin KTH0-1S*	The KTH0-1S can have an impact on sensitive cells, causing pores formation in the membrane, resulting in cell death due to loss of essential intracellular substances	(Saelao, et al. 2017)
Bacteriocin produced by Lactobacillus paracasei ZFM54	*Bacteriocin Paracin 54*	The treatment with Paracin 54 enhanced the permeability of the cell membrane, damaged the cell membrane, and led to electrolyte outflow.	(Zhu, et al. 2021)

Name	Active Components	Mechanisms	References
Bacteriocins			
Bacteriocin produced by Lactobacillus plantarum 163	*Plantaricin 827*	Its antibacterial mechanism increased the cell membrane permeability and integrity, resulting in the leakage of K+ and changes in cell morphology.	(Zhao, et al. 2022)
Bacteriocin produced by Lactococcus lactis subsp. lactis. and Lactobacillus reuteri	*Nisin and reuterin*	The combination of nisin and reuterin may change the permeability of the outer membrane and cause a lethal effect.	(Yehia, et al. 2022)
Probiotics			
Lactobacillus plantarum KU200656	NR	It can inhibit the adherence of pathogens by competing for nutrition and host intestinal cell-binding sites, e.g., receptor exclusion. bolites (e.g. hydrogen peroxide), and proteinaceous compounds.	(Lee, et al. 2021)
Lactobacillus acidophilus and *Lactobacillus casei*	NR	They produce antimicrobial components that can inhibit the growth and eliminate of the *MRSA* cells.	(Karska-Wysocki, et al. 2010)

NR: not reported.

CONCLUSION

According to our review, the most effective EO against *MRSA* was a liposome containing cinnamon oil, which resulted in a significant decrease in *MRSA* populations. Additionally, essential oils from *Cinnamomum zeylanicum*, *Syzygium aromaticum*, *Cuminum cyminum*, allicin, *glabrol, clove buds,* and *backhousia citriodora* have shown significant effectiveness against *MRSA*. Another potential solution against *MRSA* is the use of bacteriophages. Based on our review, promising phage compounds include *S. aureus* phage containing *CHAPLysGH15* and *LysGH15* and *phage SA11 endolysin*. Bacteriocins have also shown promise in combatting *MRSA*. Bacteriocins such as *Enterocin AS-48, Pentocin JL-1*, bacteriocin-producing *Pseudomonas aeruginosa TA6*, and bacteriocin produced by *S. pasteuri RSP-1* were effective against *S. aureus*. Probiotics have also shown antimicrobial properties against *MRSA*. *Streptomyces griseus*, *Pediococcus acidilactici* strains *A11 and C12, Lactococcus lactis*, and *Lactobionic acid* are among the most effective probiotics against *MRSA*. In the fight against *MRSA*, the combination of above-mentioned antibacterials has also shown promising results. These natural compounds and microorganisms possess unique mechanisms of action that can effectively target and eliminate *MRSA* cells. Furthermore, their use in combination with other antimicrobial agents including chemicals can enhance their efficacy, providing a multi-hurdle approach in combatting antibiotic-resistant bacteria. However, in food matrices, the results might be different from in vitro experiments because natural compounds can interact with food compounds like proteins and lipids, potentially reducing the availability of the natural compound as an antimicrobial agent. Additionally, processing steps can diminish the antimicrobial activity of such compounds. Furthermore, before the application of natural antimicrobials in food products, health and safety risks associated with them should be thoroughly assessed. The impact of EOs on the organoleptic characteristics of food should also be taken into account, as they may have negative effects. Nevertheless, the application of nanotechnology can mitigate these effects.

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Author's Contribution:

Z.A. made contributions to conception and design of the study, was involved in data collection and drafting the manuscript. G.S. revised the manuscript critically and together with Z.A., M.H., and A.A. prepared the final draft of the manuscript etc. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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POTENTIAL OF DIFFERENT MYCOTOXIN ADSORBENTS UNDER *IN VITRO* CONDITIONS

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Abstract

Mycotoxins are a large and chemically diverse group of toxic secondary metabolites. Regarding their prevalence in animal feed and the effect on animal health, the biggest problems in terms of safety and economic losses are caused by aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone. Adsorbents are substances that are added to food contaminated with mycotoxins, in order to bind them in the gastrointestinal tract and thereby prevent or reduce their effect. The aim of this study was to examine the possibility of using pyrophyllite as a mineral adsorbent, as well as preparations made of ground peach pits of different particle sizes as organic adsorbents, for adsorption of deoxynivalenol and ochratoxin A. Mycotoxin adsorption experiments were performed *in vitro* in electrolyte solutions at pH 3 and 7. The adsorption efficiency of the adsorbent was expressed as adsorption index. Pyrophyllite had adsorption index of 13.47% for ochratoxin A at pH 3, while at pH 7, as well as for deoxynivalenol, the same mycotoxin produced a negligible degree of adsorption. Ground peach stones (of larger diameter, d = 0.1 mm) had considerable adsorption rates for ochratoxin A at pH 3 (34.41%) and deoxynivalenol at pH 7 (18.57%). The values were similar for smaller diameter (d < 0.1 mm) for ochratoxin A at pH 3 (42.71%) and deoxynivalenol at pH 7 (20.11%). The obtained results suggest that the potential of the preparation of ground peach stones for the adsorption of tested mycotoxins is higher compared to the potential of pyrophyllite, but there are differences in their efficiency depending on the pH value of the adsorption environment.

Key words: ochratoxin A, deoxynivalenol, adsorbents, pyrophyllite, peach stones

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POTENCIJAL RAZLIČITIH ADSORBENATA MIKOTOKSINA U *IN VITRO* USLOVIMA

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Kratak sadržaj

Mikotoksini su relativno velika i hemijski raznolika grupa toksičnih sekundarnih metabolita. Uzimajući u obzir prevalencu u hrani za životinje i efekat na zdravlje životinja, najveće bezbednosne i ekonomske probleme predstavljaju aflatoksini, fumonizini, ohratoksini, trihoteceni i zearalenon. Adsorbenti predstavljaju materije koje se dodaju hrani kontaminiranoj mikotoksinima, kako bi ih u gastrointestinalnim traktu vezivali i time sprečili ili umanjili njihovo dejstvo. Cilj ovog rada je da se ispita mogućnost primene pirofilita kao predstavnika mineralnih, kao i preparata mlevenih koštica breskve različitih veličina čestica kao predstavnika organskih adsorbenata za adsorpciju deoksinivalenola i ohratoksina A. Eksperimenti adsorpcije mikotoksina su izvođeni *in vitro* u rastvorima elektrolita na pH 3 i 7. Efikasnost adsorpcije adsorbenta je izražena kao indeks adsorpcije. Rezultati su ukazali da pirofilit ima vrednost indeksa adsorpcije od 13,47% za ohratoksin A pri pH 3, dok je za isti mikotoksin pri pH 7, kao i za deoksinivalenol, utvrđen zanemarljiv nivo adsorpcije. Mlevene koštice breskve većeg dijametra (d = 0,1 mm) su pokazale odgovarajuće indekse adsorpcije za ohratoksin A pri pH 3 (34,41%) i deoksinivalenol pri pH 7 (18,57%), dok su mlevene koštice breskve manjeg dijametra (d < 0,1 mm) pokazale slične vrednosti indeksa adsorpcije za ohratoksin A pri pH 3 (42,71%) i za deoksinivalenol pri pH 7 (20,11%). Iz dobijenih rezultata se može zaključiti da je potencijal preparata mlevenih koštica breskve za adsorpciju ispitanih mikotoksina veći u odnosu na potencijal pirofilita, ali da postoje razlike u njihovoj efikasnosti u zavisnosti od pH vrednosti sredine u kojoj se adsorpcija odvija.

Ključne reči: ohratoksin A, deoksinivalenol, adsorbenti, pirofilit, koštice breskve

INTRODUCTION

Agriculture is becoming increasingly important due to the rise in world population and urbanization. This implies a rapid growth and boost of livestock production, resulting in the safety of animal feed becoming even more significant considering the potential threat of hazards reaching the human food chain. Among the undesirable substances, according to Directive 2002/32/EC (European Commission, 2002), mycotoxins are considered one of the greatest hazards in feed raw materials (Santos Pereira et al., 2019). Among other mycotoxins, deoxynivalenol (DON) and ochratoxin A (OTA) certainly represent the most important causes of diseases in animals. Namely, global economic losses caused by DON are in the range of about one billion dollars per year. The Food and Agriculture Organization and the World Health Organization identified it as one of the most dangerous food contaminants as early as 1973 (Neme and Mohammed, 2017). This mycotoxin mainly contaminates agricultural crops such as corn, wheat, and barley, and has immunogenic, carcinogenic, and teratogenic effects in animals. It is also detected in animal products such as meat, eggs, and milk, and poses a danger to human health (Yao and Long, 2020). OTA causes great economic losses to animal husbandry, as the intake of contaminated feed can significantly impair animal health and safety of animal products (Battacone et al., 2010). It can be found in a wide range of agricultural and livestock products, and in processed foods as well, while human exposure to this mycotoxin is mainly attributed to contaminated grains (Abrunhosa et al., 2010). Juodeikiene et al. (2012) stated three possible solutions for avoiding the harmful effect of mycotoxin contamination of both food and feed. They include the following: prevention of contamination, decontamination of mycotoxin-containing food and feed, and inhibition of mycotoxin absorption into the digestive tract. Nevertheless, if mycotoxin contamination already exists, application of decontamination methods is advised (Jouany, 2007). Various methods for mycotoxin reduction were studied, including the application of irradiation, thermal or microwave heating, ozone, chemical compounds, and microbials (Binder and Binder, 2004; Bullerman and Bianchini, 2007; Herzallah et al., 2008; Garg et al., 2013; Krstović et al., 2021). Moreover, the use of existing feed additives, such as enzymes showed a potential for degradation of some mycotoxins (Jakšić et al., 2022). Finally, measures can be applied for inhibition of mycotoxin absorption in the gastrointestinal tract, e.g., the use of adsorbents to reduce the bioavailability of mycotoxins in the digestive tract of animals (Abdel-Wahhab and Kholif, 2010). The use of adsorbents is one of the most suitable options for treatment of mycotoxins in practice (Li et al., 2018). In general, the adsorption method includes both physical and chemical force,

which can not only reduce the toxic impact of mycotoxins, but also enable the avoidance of toxic residues, thus becoming the most commonly applied method of protecting animals from mycotoxins (Li et al., 2018). Adsorbents can be used as single formulations, but are recently combined for better efficiency, usually as mineral-organic adsorbent mixture (Nešić et al., 2020).

The aim of this study was to examine the possibility of using two types of mycotoxin adsorbents. The first is pyrophyllite, a non-metallic aluminosilicate mineral that has adsorptive properties, primarily for ions of heavy metals and colors from aqueous solutions. The second adsorbent included two preparations of ground peach stones of different particle sizes. This type of adsorbent is organic, its waste biomass of agro-industrial source, and as an economically and ecologically profitable material, it is generally considered cheaper than mineral (inorganic) adsorbents. The adsorptive capacity of these preparations was tested for the adsorption of DON and OTA under *in vitro* conditions.

MATERIAL AND METHODS

Adsorbents

Adsorption potential test was performed for two preparations. Pyrophyllite, which belongs to the group of non-metallic aluminosilicate minerals, was selected as a potential inorganic adsorbent. Its use is already permitted in animal feed at up to 2% to prevent ruminal acidosis in ruminants (US Food and Drug Administration, 2019; Adamović et al., 2020). Another potential adsorbent is a preparation of ground peach stones (Lopičić et al., 2013). It was prepared in two fractions: larger (particle size d = 0.1 mm) and smaller (particle size d < 0.1 mm) fraction. The preparations were not commercially available. All preparations were prepared by and obtained from the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade, Serbia.

Mycotoxin removal procedure in vitro

To simulate *in vivo* conditions, where mycotoxin adsorption is performed on adsorbents, mycotoxin adsorption experiments were performed *in vitro* in solutions at pH 3 and 7. These pH values are expected in the digestive tract of monogastric animals and humans. The crude extract of DON, produced in house on wet maize kernels after artificial inoculation with toxigenic *Fusarium graminearum* (Krstović et al., 2018), as well as the standard solution of OTA (Sigma, St. Louis, MO, USA) were used for the adsorption experiments. 90 mg

of adsorbent was weighed into glass tubes using Teflon stoppers (volume of 20 cm^3), and then 10 mL of 0.1 M KH2PO4 (13.609 g KH2PO4 in 1000 mL of water, pH set to pH = 3 and pH = 7) was added. After that, a volume of 100 µL of crude toxin solution (c = 300 µg/mL) was added in order to obtain the final concentration of 3 µg/ml (crude toxin to adsorbent ratio was 1:3000). Mixing magnets were added to the tubes and then placed in an incubation system at 37 °C for 120 minutes with continuous mixing. The control was set up in the same way, without adsorbent. After the contact of the adsorbent and the mycotoxin was completed, the solution was filtered through quantitative filter paper for slow filtration and then additionally through nylon micro syringe filters with a porosity of 0.2 µm. The solutions prepared in this way were analyzed for HPLC for the mycotoxins' contents. All experiments were performed in duplicate. The adsorption efficiency of the natural adsorbent is expressed as an adsorption index, where:

Ci – mycotoxin initial concentration,

Ceq – mycotoxin concentration at equilibrium.

Adsorption index, % = [((Ci – Ceq)/Ci)] × 100

HPLC analysis

DON and OTA determinations were carried out on a 1260 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) using photodiode (DAD) and fluorescence (FLD) detectors (Agilent Technologies, USA) and a Hypersil ODS (150 x 4.6 mm i.e., particle size 5 µm) column (Agilent Technologies, USA). Stock calibration solutions at a concentration of 0.1 mg/mL were prepared in a mixture of ethyl acetate and methanol (19:1, v/v) for DON (Sigma, St. Louis, MO, USA), and in acetonitrile for OTA (Sigma, St. Louis, MO, USA). Working calibrant solutions were prepared by measuring the appropriate volume of the stock solution, evaporating to dryness under a stream of nitrogen at 50 °C and dissolving in the appropriate volume of the mobile phase. The stock solutions were stored at a temperature of -18 °C, while the working solutions were stored in a refrigerator at a temperature of 4 – 8 °C. HPLC conditions for DON determination were set as proposed by Abramović et al. (2005) and for OTA a method by Sugita-Konishi et al. (2006) was used. All analyses were done in duplicate. A volume of 20 µL of solution obtained after adsorption was injected into a HPLC system. The mobile phase consisted of an isocratic mixture of acetonitrile and water (HPLC grade, Sigma, St. Louis, MO, USA). For the determination of DON, the ratio of the solvents was the following: acetonitrile: water (16:84, *v/v*), and in the case of OTA, this ratio

acetonitrile: water was (50:50, *v/v*) and 1% of acetic acid was added (HPLC purity, Fisher Scientific, USA). In both cases, a mobile phase flow rate of 0.8 mL/min was applied. Before use, the mobile phase was filtered through regenerated cellulose membrane filters (0.45 μm) (Agilent Technologies, USA). DON was detected using a DAD detector at a wavelength of λ = 220 nm, for the detection of OTA, FLD detector was used at wavelengths of λ_{ex} = 333 nm and λ_{em} = 470 nm. The identification of DON and OTA was performed by comparing the retention times and spectra of the standards with the retention times and spectra of the samples.

RESULTS

The results of mycotoxin adsorption using the *in vitro* method are shown in tables 1-3.

Table 1. Results of mycotoxin adsorption using pyrophyllite, n = 2

		DON	**OTA**
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.53	0.17
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2945 ± 23	2569 ± 21
	A, %	0.03	13.47
pH 7	Ci, ng/mL	2978 ± 31	2980 ± 11
	Ceq, ng/mL	2974 ± 13	2948 ± 32
	A, %	0.15	1.1

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

Table 2. Results of mycotoxin adsorption using the preparation of ground peach stones (GPS), particle size d = 0.1 mm, n = 2

		DON	OTA
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.5	0.2
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2940 ± 56	1948 ± 89
	A, %	0.20	34.41
pH 7	Ci, ng/ml	2978 ± 31	2980 ± 11
	Ceq, ng/ml	2425 ± 38	2972 ± 12
	A, %	18.57	0.29

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

Table 3. Results of mycotoxin adsorption using the preparation of ground peach stones (GPS), particle size d < 0.1 mm, n = 2

		DON	OTA
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.5	0.2
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2941 ± 28	1701 ± 98
	A, %	0.19	42.71
pH 7	Ci, ng/mL	2978 ± 31	2980 ± 11
	Ceq, ng/mL	2379 ± 89	2897 ± 56
	A, %	20.11	2.80

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

The adsorption index of DON was below 1% using pyrophyllite for both pH values, as well as in the case of using the preparation of ground peach stones at pH = 3 (Figure 1). As for the preparation of ground peach stones

at pH = 7, adsorption index values of 18.57% (larger fraction) and 20.11% (smaller fraction) were obtained. This indicates that there is a certain potential of the ground peach stone preparation for DON adsorption at pH = 7, while the adsorption potential of pyrophyllite has not been proven.

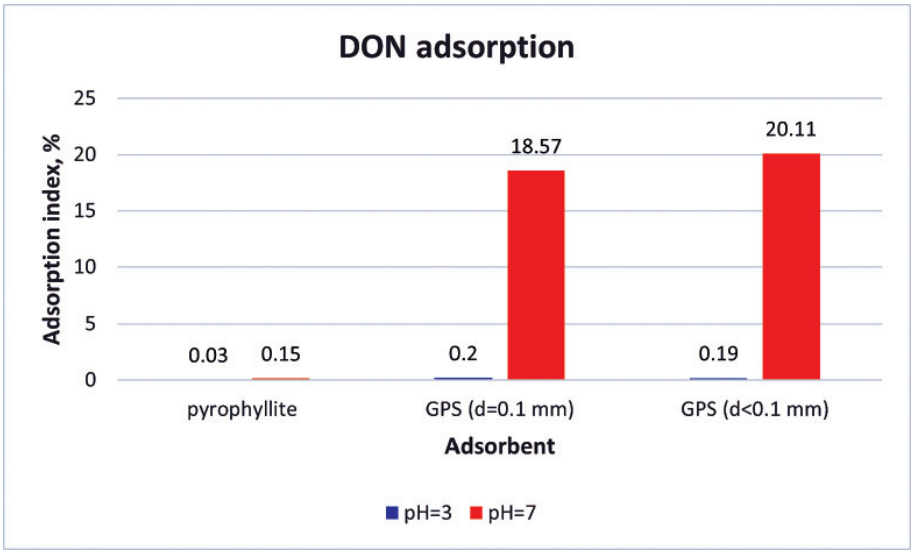

Figure 1. Comparative overview of the adsorption index of ground peach stones (GPS) and pyrophyllite for DON adsorption

When it comes to the adsorption index of pyrophyllite for OTA, it was affected by the pH value (Figure 2). Namely, at pH=3, the adsorption index was found to be 13.47%, while at pH = 7, it was as low as 1.1%. However, using the preparation of ground peach stones, these differences in the adsorption index were opposing compared to the application of pyrophyllite, where high values of the adsorption index of 34.41% (larger fraction) and 42.71% (smaller fraction) were found at pH = 3, and low values (0.29% and 2.80%) at pH = 7. These results indicate that the potential of the preparation of ground peach stones for OTA adsorption is higher compared to the potential of pyrophyllite, but there are still differences in their efficiency depending on the pH value of the environment in which the adsorption takes place.

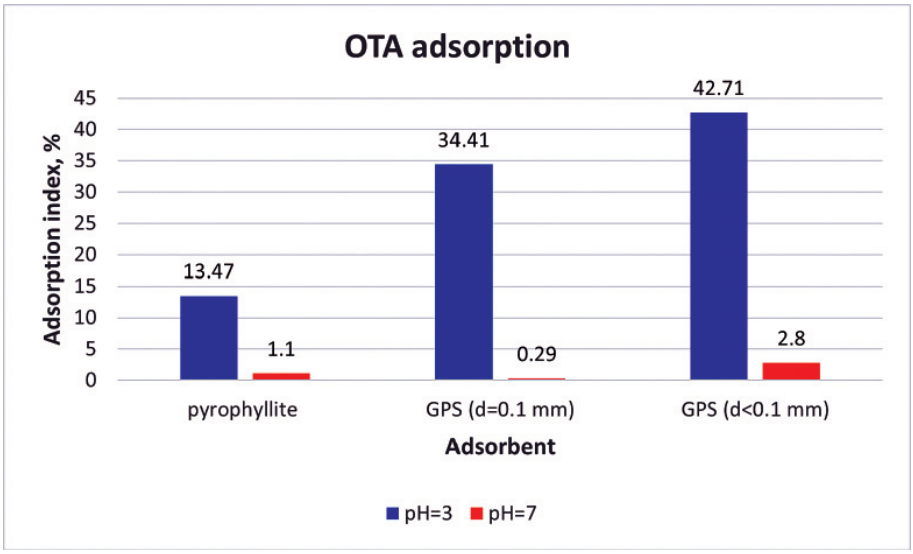

Figure 2. Comparative overview of the adsorption index of ground peach stones (GPS) and pyrophyllite for OTA adsorption

DISCUSSION

Pyrophyllite is already a recognized adsorbent of various substances such as cationic (due to negative hydrophilic surface sites) and anionic (after the addition of trivalent aluminium cation) dyes (Gücek et al., 2005). It is also known for its ability to bind ions of heavy metals such as cobalt (98.97%), lead (97.53%), nickel (98.87%) and cadmium (98.65%) from aqueous solution (maximum adsorption in optimized conditions) (Panda et al., 2018). Furthermore, in the research conducted by Murtić et al. (2020), it was demonstrated that the application of pyrophyllite, due to its adsorbent and ion exchange capabilities can reduce the use of mineral fertilizers in the production of lettuce without adverse effects on its yield and quality, in the amount of 25% or 50% of the recommended amount of fertilizer. However, despite the results of the aforementioned research, as well as the proven adsorption capacity of numerous other aluminosilicates against mycotoxins, literature data on the adsorption of mycotoxins, specifically by pyrophyllite, is very scarce. In contrast, the potential of peach stones to adsorb mycotoxins has been studied more extensively. Hence, in the research by Adamović et al. (2013) the adsorption of the following six mycotoxins was tested: AFB1, zearalenone, diacetoxyscirpenol (DAS), T-2 toxin, OTA and DON using the *in vitro* method. Similar to our study, the adsorption index was tested at two pH values, pH = 3.0 and 6.9.

Peach stones showed the highest affinity to aflatoxin (80.00% and 73.30%) and the lowest to DAS (0% at both pH values). When it comes to OTA and DON, a higher adsorption capacity was demonstrated in comparison with our research, given that the adsorption index at pH=3.0 and 6.9 were 66.67% and 50% for OTA, and 25% and 50% for DON, respectively. However, there were certain differences in the experimental design that could produce a different result (the ratio of individual mycotoxins and adsorbents in this research was 1:5000 and the particle size of the investigated biomass < 100 μm). Comparable research was conducted by Lopičić et al. (2013), at pH values of 3.0 and 7.0. Similar to the previous study, the best adsorption index of peach stones was obtained for aflatoxin (58.82% at both pH values), the poorest for DAS (0% and 16.67%), while for OTA it was 66.67% and 19.98% and 23.08% and 39.97% for DON, at pH 3.0 and 7.0, respectively. It is interesting to point out that in both studies, in addition to the unmodified, modified peach stones (activated with 1 M HCl) were used, and they achieved a somewhat different, weaker result compared to the unmodified. Furthermore, in the research by Bočarov-Stančić et al. (2011), the adsorption capacities of mineral adsorbents bentonite, diatomite and zeolite against aflatoxin, DAS, OTA, and DON were investigated. Like in the previous research, the highest adsorption index was obtained for aflatoxins (at pH 3.0 and 6.9: bentonite 96.90%; zeolite 95.50% and diatomite 95%) which is slightly higher compared to organic adsorbents, while the weakest was obtained for DAS (0% in all adsorbents). However, it is noted that organic adsorbents were more effective for OTA and DON. With OTA, only diatomite had adsorption properties (66.67%) at pH = 3. On the other hand, at pH = 6.9, as well as for bentonite and zeolite at both pH values, the adsorption index was 0%. Considering DON, mineral adsorbents showed affinity at pH = 3, bentonite and zeolite 50% and diatomite 25%, whereas at pH = 7 they did not display any affinity (0%). These results may lead to a conclusion that mineral adsorbents are more effective when it comes to aflatoxins, while organic adsorbents are more successful for OTA and DON adsorption. This was confirmed by the results of our research, where pyrophyllite was used as a mineral adsorbent. This adsorbent had an effect (13.47%) only for OTA at pH = 3, while it was ineffective in other cases. Ground peach stones were more efficient, as a larger diameter showed the degree of adsorption for OTA at pH = 3 (34.41%) and DON at pH 7 (18.57%), and the smaller diameter similarly, for OTA at pH = 3 (42.71%) and for DON at pH = 7 (20.11%). However, it is certainly necessary to prove this result under *in vivo* conditions.

The obtained results in our and similar research indicate that the degree of binding depends primarily on the type of adsorbent (mineral or organic), and

on individual types within these groups, as well as on the mycotoxin. These characteristics should be considered when creating formulations for use in practice, and certain adsorbents should be used depending on the conditions on the farm and occurrence of certain types of mycotoxins. In the future, formulations containing several types of adsorbents need to be tested, in order to enable action against several types of mycotoxins or use one of them that has the widest spectrum of adsorption capacity when different types of mycotoxins are involved.

CONCLUSION

The results of this study point to several conclusions. Namely, the potential of the preparation of ground peach stones for the adsorption of DON at pH = 7 was found, while its application at pH=3, the adsorption potential was not confirmed. The adsorption potential of pyrophyllite was not established at any of the pH values to which it was applied. The potential of the preparation of ground peach stones for OTA adsorption is higher compared to the potential of pyrophyllite, but there are differences in their efficiency depending on the pH value of the environment in which the adsorption takes place. Like with other *in vitro* research, this research cannot fully simulate conditions in the gastrointestinal tract of animals, so further *in vivo* experiments are necessary in order to evaluate the effectiveness of these materials as mycotoxin adsorbents.

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Author's Contribution:

S.K. basic idea, conception and design; D.G. acquisition of data; J.K. interpretation of results, manuscript revision; M.D. acquisition of data, drafting the manuscript; I.J. conception and design, final approval.

Competing interest

The author(s) declare that they have no competing interests.

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ISOLATION, MOLECULAR IDENTIFICATION AND ANTIBIOTIC SENSITIVITY OF *SALMONELLA* FROM BUFFALO FECES IN SYLHET DISTRICT OF BANGLADESH

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Abstract

Salmonella is a widely distributed foodborne pathogen affecting humans and animals around the globe. This cross-sectional bacteriological study was aimed at isolation of *Salmonella* from fecal samples of buffalos in Sylhet district (Upazilas Jaintapur and Fenchuganj) of Bangladesh, their molecular confirmation, and learning their antibiotic sensitivity patterns. A total of 334 fecal samples were collected using a simple random sampling technique. Standard conventional bacteriological culture and biochemical tests were done to isolate and confirm *Salmonella*. The isolates were confirmed with polymerase chain reaction (PCR). In this study, *Salmonella* was isolated from 56 samples (16.77%) using bacteriological culture methods and biochemical tests, and all isolates were confirmed in PCR tests. The prevalence of *Salmonella* was estimated to be 17.57% and 13.93% in Jaintapur and Fenchuganj Upazilas, respectively. Buffalo calves under one year of age had a higher prevalence (24.32%) than older animals aged 1 - 2 (18.62%), 2 - 4 (13.25%), and > 4 years (10.67%). Furthermore, the prevalence was substantially higher in diarrheic animals (72.22%) than in their healthy counterparts (13.60%). It did not vary significantly ($p > 0.05$) between animals from Jaintapur and Fenchuganj Upazilas. Likewise, no significant difference ($p > 0.05$) in *Salmonella* isolates was detected between different age groups. The results revealed that the isolation rate of *Salmonella* was significantly ($p < 0.05$) higher in female and diarrheic animals.

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The most effective antibiotics against the majority of *Salmonella* isolates were gentamycin (100%), levofloxacin (100%), and ciprofloxacin (76.67%). On the other hand, *Salmonella* isolates were highly resistant to tetracycline (100%), ampicillin (87.5%), and streptomycin (78.6%). Pathogenic microorganisms in feces are a potential risk to public health due to environmental and animal food contamination. Therefore, infection control and establishing strategic antibiotic therapy should be a priority.

Key words: Bangladesh, buffalo feces, *Salmonella*, *inv*A gene, antibiotics sensitivity

IZOLACIJA, MOLEKULARNA IDENTIFIKACIJA I ANTIBIOTSKA OSETLJIVOST *SALMONELLA* IZ BIVOLJEG IZMETA U OKRUGU SILHET, BANGLADEŠ

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Kratak sadržaj

Salmonella je široko rasprostranjen patogen koji se prenosi hranom i pogađa ljude i životinje širom sveta. Ova bakteriološka studija poprečnog preseka imala je za cilj izolaciju *Salmonella* iz uzoraka fecesa bivola u okrugu Silhet (distrikti Jaintapur i Fenchuganj) u Bangladešu, njihovu molekularnu potvrdu i osetljivost na antibiotike. Ukupno 334 uzorka fecesa prikupljena su jednostavnom tehnikom slučajnog uzorkovanja. Standardne konvencionalne bakteriološke metode i biohemijski testovi su urađeni da bi se izolovala i potvrdila *Salmonella*. Izolati su potvrđeni lančanom reakcijom polimeraze (PCR). U ovoj studiji *Salmonella* je izolovana iz 56 uzoraka (16,77%) metodom kultivacije i biohemijskim testovima, a svi izolati su potvrđeni i PCR testovima. Prevalencija *Salmonella* je iznosila 17,57% i 13,93% u Jaintapuru i Fenchuganj distriktu. Mladunčad bivola mlađih od jedne godine imala

su veću prevalenciju (24,32%) od starijih životinja uzrasta 1 - 2 (18,62%), 2 - 4 godine (13,25%) i > 4 godine (10,67%). Osim toga, prevalencija je bila znatno veća kod životinja sa dijarejom (72,22%) nego kod zdravih životinja (13,60%). Prevalencija nije značajno varirala ($p > 0,05$) između životinja iz Jaintapura i Fenchuganj distrikta. Isto tako, nije otkrivena značajna razlika ($p > 0,05$) u izolatima *Salmonella* između različitih starosnih grupa. Rezultati su otkrili da je stopa izolacije *Salmonella* značajno ($p < 0,05$) veća kod ženki i životinja sa dijarejom. Najefikasniji antibiotici protiv većine izolata *Salmonella* bili su gentamicin (100%), levofloksacin (100%) i ciprofloksacin (76,67%). S druge strane, izolati *Salmonella* su bili visoko otporni na tetraciklin (100%), ampicilin (87,5%) i streptomicin (78,6%). Patogeni mikroorganizmi u fecesu predstavljaju potencijalni rizik za javno zdravlje zbog kontaminacije životne sredine i hrane za životinje. Stoga, kontrola infekcije i uspostavljanje strateške antibiotske terapije treba da budu prioritet.

Ključne reči: Bangladeš, bivolji feces, *Salmonella*, *inv*A gen, antibiotska osetljivost

INTRODUCTION

Salmonella, a genus of the family *Enterobacteriaceae*, is a major cause of foodborne disease of public health significance both in developed and developing countries (Gebeyehu et al., 2022). All across the world, pathogens have been isolated from the feces of animals (Sabur et al., 2021). Salmonellosis, the clinical form of *Salmonella* infection, is a costly disease to livestock producers on account of mortality, treatment expenses, reduced milk yield, and weight loss/decreased weight gain within the herd (Callaway et al., 2005). By producing high-quality milk, meat, and farmyard waste, buffalo ranching contributes significantly to the agriculture and cattle sectors in many Asian nations (Khongsai, 2020). Bangladesh has only 1.485 million indigenous buffaloes, with a more significant concentration (40%) in coastal areas and certain buffalo pockets. Smallholder farmers in Bangladesh raise a small number of imported Nili-Ravi and Murrah buffaloes and their crosses on a few fields (Samad, 2020). Pathogens that infect buffaloes and their offspring or are transferred via their products are significant because they impact milk production and total livestock performance. In buffaloes, *Salmonella* is a major pathogen causing calf diarrhea (Khongsai, 2020; Ribeiro et al., 2000), leading to early-age

calf mortality (Rana et al., 2012) and a foremost pathogen transmitted through animal products. *Salmonella enterica* subsp. *enterica* can cause infections with a variety of clinical signs, as well as systemic infections characterized by diarrhea and septicemia, and, in extreme cases, death.

Antimicrobial resistance (AMR) is now recognized as one of the most serious global threats to human health in the 21st century (Morehead and Scarbrough, 2018; Tacconelli et al., 2018a; Tacconelli et al., 2018b) and a decline in the rate of new antibiotic development (Luepke and Mohr, 2017). It causes a considerable risk of death and economic hardship all over the world. However, impoverished nations are more harmed due to extensive antibiotic abuse, non-human antibiotic usage, poor medication quality, and insufficient surveillance (Ayukekbong et al., 2017; Van Boeckel et al., 2019). Bangladesh, a Southeast Asian developing country with a high level of AMR, poses a regional and global concern. Several studies have found irrational antibiotic prescribing by doctors and veterinarians, a patient habit of self-medication, and indiscriminate antibiotic usage in agriculture and farming in various sections of the nation (Biswas et al., 2014; Sutradhar, 2014).

Salmonella species with single and multidrug resistance are becoming more common (Liu et al., 2010). Salmonellosis is treated with a variety of antibiotics. Fluoroquinolones and third generation cephalosporins are the most often prescribed antibiotics to treat salmonellosis. Chloramphenicol, Ampicillin, Amoxicillin, and trimethoprim-sulfamethoxazole are some of the older antibiotics occasionally used as alternatives (Ayukekbong et al., 2017). Antimicrobial resistance in *Salmonella* has been observed to be on the rise in several developing and developed nations (Schmidt et al., 2018).

In some upazilas of the Sylhet region, the farmers depend upon the buffaloes primarily for draught and meat purposes, an integral part of the farming system in this area. Buffalo is economically significant in this region and could be considered a "small tractor" for farmers, and its meat and milk are nutritionally rich. Despite their great contribution to the socio-economy, they are still undervalued. Diseases are one of the most significant constraints for buffalo production, which decrease milk and meat production and reduce farmer's income. In addition, the status of *Salmonella* in buffalo populations in Sylhet district is unknown. Therefore, this study aimed to isolate and identify *Salmonella* from buffalo feces by conventional bacteriological, biochemical, and molecular methods and to determine the antibiotic sensitivity of *Salmonella* isolates.

MATERIAL AND METHODS

Study Area and Sample Collection

For this study, Fenchuganj and Jaintapur Upazilas of Sylhet District of Bangladesh were purposively selected due to their relativeness of larger buffalo population and milk production. The sampling units for the study were buffalo of different ages. A total of 334 fecal samples of buffaloes (112 from Fenchuganj and 222 from Jaintapur) of Sylhet District constituted the study population, which was selected by using a simple random sampling method. The study was conducted for a period of one year (July 2021 to June 2022). Feces samples were collected directly from the rectum and freshly voided feces from buffalo. The samples were placed in a labeled clean plastic container and immediately transferred to the laboratory for further analysis.

Isolation and identification of Salmonella

Conventional bacteriological methods were used to isolate the *Salmonella* from feces samples. The samples were inoculated into buffered peptone water (BPW), incubated at 37°C for 24 hours, and 0.5 mL was transferred to 10 mL Tetrathionate Broth (Merck) and incubated at 37°C for 24 hours. Following enrichment, a loopful of samples were streaked on xylose-lysine deoxycholate (XLD) agar and *Salmonella – Shigella* (SS) agar plates and incubated at 37 °C for 24 h. Biochemical confirmation of *Salmonella* was performed using TSI agar, Urea agar, Simmon's citrate agar, methyl red, and Voges Proskauer broth.

Bacterial genomic DNA Extraction and PCR assay

The DNA extraction was performed according to the manufacturer's instructions using the Addprep genomic DNA extraction kit (Addbio Inc. Ltd., Korea). These eluted DNA samples were stored at -80 °C until further analysis. The PCR analysis was performed to detect the *Salmonella* invasion gene (*invA* gene) according to the manufacturer's instructions (Addbio Inc. Ltd., Korea). PCR assay performed in the thermal cycler TC1000G PCR System® (DLAB Scientific Inc., USA) with a heated lid. The cycling conditions included 50 °C for 3 minutes (UDG Reaction), 95 °C for 10 minutes (Initial Denaturation), 35 cycles of 95 °C for 30 seconds (denaturation), 68 °C for 45 seconds (annealing), and 72 °C for 5 minutes for final extension (Khan et al., 2021). For the detection of *Salmonella*, the primers of invS-F (5'-TAA TGCCAGACGAAAGAGCGT-3') and invS-R (5'-GATATTGGTGTTTATGGG GTCGTT-3') were

used (Khan et al., 2021). All reaction mixtures, including the negative control and *Salmonella* positive DNA, were tested in duplicate in the same run of PCR assay. PCR products were analyzed on 1.8% agarose gels stained with RedSafe™ (iNtRON Biotechnology, Korea) Nucleic Acid Staining Solution (20,000×), photographed, and stored as a digital image.

Determination of Antibiotic sensitivity patterns

The *in vitro* antibiotic sensitivity test was determined by the standard disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI, 2020). The antibiotic discs used in the present study were Ampicillin (AMP, 10 µg), Amoxicillin/Clavulanic Acid (AMC, 30 µg), Gentamycin (CN, 10 µg), Streptomycin (S, 10 µg), Cefixime (CFM, 5 µg), Ceftriaxone (CRO, 30 µg), Azithromycin (AZM, 15 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Colistin sulfate (CT, 10 µg), Tetracycline (TE, 10 µg), Sulfamethoxazole-trimethoprim (SXT, 25 µg) all from Oxoid company, UK. After incubation, each disc's diameter of the clear zones produced by antibacterial inhibition of bacterial growth was measured to the nearest millimeter and then classified as resistant, intermediate, or susceptible using a published interpretive chart of Clinical and Laboratory Standards Institute (CLSI,2020).

Statistical analysis

Microsoft Excel Office 2010 was used for descriptive statistics. Chi-square tests were used to assess the significance of differences in prevalence between age, sex, and health status of the animals. P values less than 0.05 were considered significant using Chi-square tests (SPSS Inc., Chicago, IL, USA).

RESULTS

Salmonella was isolated from 56 (16.77%) out of 334 examined samples, of which 39 (17.57%) and 17 (13.93%) were from feces of Jaintapur and Fenchuganj upazila, respectively (Table 1). The prevalence of *Salmonella* was higher in buffalo calves aged less than one year (24.32%); although the difference was not statistically significant ($p = 0.11$) (Table 2). Furthermore, the isolation rate was relatively higher in animals aged 1 - 2 years (18.62%) than in the groups of 4 years and above four years (Table 2).

Table 1: Overall prevalence of *Salmonella* in buffalo feces and its association with study area

Study area	Number of samples tested	Tests name					
		Culture positive	Prevalence	Biochemical Test positive	Prevalence	PCR positive	Prevalence
Jaintapur	222	45	20.27%	39	17.57%	39	17.57%
Fenchuganj	122	19	15.57%	17	13.93%	17	13.93%
Total	334	64	19.16%	56	16.77%	56	16.77%

Table 2: Association of the prevalence of *Salmonella* with age of buffalo populations

Age of animals	No. of animals examined	No. of positive fecal samples	Prevalence	P-value
Up to 1 year	74	18	24.32%	0.11
1 – 2 years	102	19	18.62%	
2 - 4 years	83	11	13.25%	
> 4 years	75	8	10.67%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

It was observed that out of the total 334 feces samples collected from two different upazilas, the female buffaloes showed a higher prevalence (21.56%) of *Salmonella* in their fecal samples (Table 3). All suspected *Salmonella* colonies underwent specific biochemical tests such as indole formation, methyl red and Voges Proskauer reaction, citrate utilization, and triple sugar iron agar due to their cultural and morphological characteristics. Sixty-four of the suspected *Salmonella* colonies had 56 confirmed colonies by biochemical test results. Most of the *Salmonella* isolates showed fermentation of glucose, gas

production from glucose, H_2S formation, but none of the isolates showed either lactose or sucrose. The results showed that the prevalence rate of *Salmonella* was statistically significant (p = 0.01). In this study, the *Salmonella* isolation rate was categorized based on the health condition of buffalo populations. The isolation rate of *Salmonella* was higher in diarrheic buffalo fecal samples (72.22%) compared with apparently healthy animals (13.60%). The result revealed statistically significant differences (p = 0.01) (Table 4).

Table 3: Association of *Salmonella* isolation rate with the sex of animals

Sex of animals	No. of animals examined	No. of positive fecal samples	Prevalence	*p*-value
Male	153	17	11.11%	0.01
Female	181	39	21.55%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

Table 4: The association of *Salmonella* isolation rate with the health status of buffalo populations

Health status of animals	No. of animals examined	No. of positive fecal samples	Prevalence	*p*-value
Apparently healthy	316	43	13.60%	< 0.01
Diarrheic	18	13	72.22%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

A total of 56 isolates of *Salmonella* were tested for commonly used 12 antibiotics from 8 groups. All the *Salmonella* isolates were found resistant to one or more antibiotics used. This antibiotic susceptibility study revealed that *Salmonella* isolates were 100%, 93.33%, and 76.67% sensitive to Gentamycin, Levofloxacin, Colistin sulfate, and Ciprofloxacin. On the other hand, isolates were 100%, 87.5%, and 78.6% resistant to Tetracycline, Ampicillin, and Streptomycin, respectively (Table 5.). All of the biochemically positive *Salmonella* isolates

(n = 56) were submitted to a PCR test using a particular primer set to identify the *invA* gene of *Salmonella*. After agarose gel electrophoresis under UV transilluminator, all the biochemically identified 56 isolates were confirmed positive for *Salmonella* and revealed *an invA* gene-specific band at 100bp (Figure 1.).

Table 5. Antibiotic susceptibility profile of *Salmonella* isolated (n = 56) from buffalo feces

Antibiotic tested	**Susceptible (%)**	**Intermediate (%)**	**Resistance (%)**
Penicillin			
Ampicillin (AMP, 10 µg)	-	7 (12.5)	49 (87.5)
Amoxicillin/clavulanic Acid (AMC, 30 µg)	30 (53.33)	15 (26.67)	11 (20)
Aminoglycosides			
Gentamicin (CN, 10 µg)	56 (100)	-	-
Streptomycin (S, 10 µg)	-	12 (21.4)	44 (78.6)
Cephalosporins			
Cefixime (CFM, 5 µg)	41 (73.21)	4 (7.14)	11 (20)
Ceftriaxone (CRO, 30 µg)	39 (70)	4 (7.14)	13 (23.21)
Macrolides			
Azithromycin (AZM, 15 µg)	39 (70)	-	17 (30)
Fluoroquinolones			
Ciprofloxacin (CIP, 5 µg)	43 (76.67)	13 (23.21)	-
Levofloxacin (LEV, 5 µg	56 (100)	-	-
Polymyxins			
Colistin Sulphate (CT, 10 µg)	52 (92.86)	-	4 (7.14)
Tetracyclines			
Tetracycline (TE, 10 µg)	-	-	56 (100)
Folate Pathway Inhibitors			
Sulfamethoxazole -Trimethoprim (SXT, 25 µg)	35 (62.5)	-	21 (37.5)

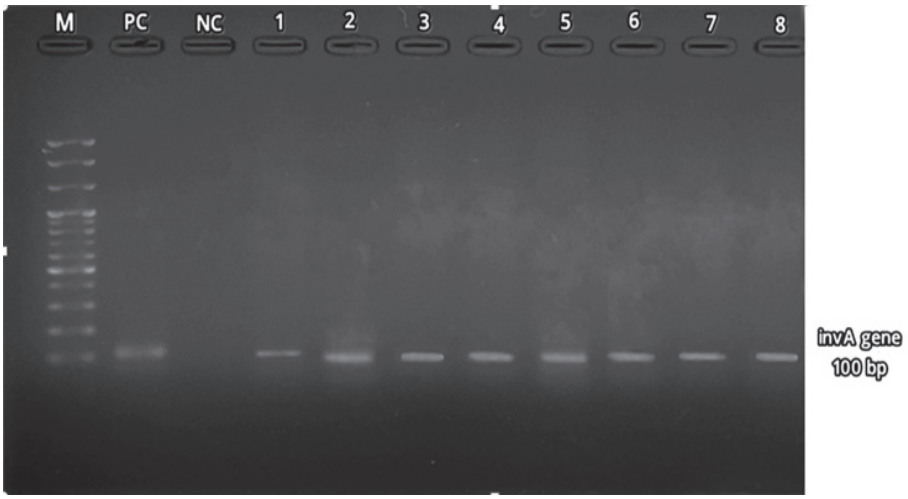

Figure 1. PCR amplification of biochemically identified *Salmonella*

DISCUSSION

In the present study, 334 buffalo fecal samples were collected from Jaintapur (222) and Fenchuganj (112) Upazilas of Sylhet district. Bacteriological examination revealed the *Salmonella* organisms from 56 of 334 buffalo fecal samples with a prevalence of 16.77% (Table 1), which is close to 11.2% (Zahran and Elbehiry, 2014). *Salmonella* in the buffalo population is a health risk for people who consume the products (meat, milk), as it has been reported that 25% of meat samples are contaminated with *Salmonella* (Abd-Elghany et al., 2022). The prevalence of *Salmonella* was higher in young (up to 1 year) and sick (diarrheic) animals, estimated to be 24.32% and 72.22%, respectively. The age-related result of the present study is consistent with the findings of other researchers (Saha et al., 2013; Mahmood et al., 2014). The findings also coincide with the results of Hunduma et al. (2010), who stated that diarrhea is a major problem in animals with a higher prevalence.

For detection of *Salmonella* genus using PCR, it was found that all PCR products, including positive control, resulted in 100 bp amplified fragment. Figure 1 revealed no amplified DNA fragments obtained from the non-*Salmonella* genus. *Salmonella* isolates produced a white band at the level of marker DNA (Figure 1). The PCR method is recommended for detecting *Salmonella* in feces samples because it is faster than culture methods (Stone et al., 1994; van der Zee and Huis in´t Veld, 2000).

According to Vella and Cuschieri (1995), *Salmonella* isolates were most frequently resistant to trimethoprim. *Salmonella* isolated from feces of dairy cattle were resistant to ampicillin (100%), streptomycin (66.7%), and tetracycline (55.56%) but susceptible to gentamicin (100%) and ciprofloxacin (91.7%) as reported by others (Khan et al., 2021). According to Wieczorek and Osek (2013), sulfamethizole resistance was found in various strains of *S.* Dublin and *S.* Enteritidis. *Salmonella* isolates are resistant to multiple antibiotics (ampicillin, streptomycin, tetracycline, and sulfamethizole). The isolates were susceptible to gentamycin (100%), levofloxacin (100%), and ciprofloxacin (76.67%) but have shown higher resistance to tetracycline, ampicillin, and streptomycin (100%, 87.5%, and 78.6%, respectively). According to Esaki et al. (2004), many *Salmonella* isolates from both healthy and ill animals were resistant to two or more antimicrobials. These microorganisms are disseminated in the environment and spread through the excrement of animals that appear to be in good health. According to reports, *S.* Typhimurium was released in a pig farm (Tanaka et al., 2014). The release was relatively high for several days following vaccination; therefore, the animals appeared to be in good health. As a result, herd infection could come even from the excrement of clinically healthy animals. Furthermore, a range of serotypes of antibiotic-resistant bacteria may exist. *Salmonella* isolates were shown to be resistant to numerous drugs in the current investigation. Tetracycline resistance was found in all *Salmonella* isolates. The results of this study revealed that *Salmonella*-infected buffalo could be effectively treated with gentamycin, Levofloxacin, and Ciprofloxacin.

CONCLUSION

It could be concluded that *Salmonella* is a significant cause of diarrhea in buffaloes with salmonellosis in Bangladesh. Therefore, a rapid and proper diagnosis could prevent harm inflicted on the livestock industry. The molecular basis of *Salmonella* identification techniques, such as using the *invA* gene-specific PCR method, could be helpful in diagnostic and research laboratories. This study suggests the strategic use of antibiotics to control *Salmonella* infections in animals.

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Author's Contribution:

MMH and MMR: Supervised and contributed to study design; BDG and RB: Carried out the laboratory experiments; SA and MSRC: Performed statistical analysis; and BDG and MMH: Wrote the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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EVALUATION OF PHYSIOLOGICAL BIOMARKERS AS POSSIBLE PREDICTIVE FACTORS AND PROGNOSIS MARKERS OF KIDNEY INJURY IN DOGS NATURALLY INFECTED WITH *LEISHMANIA INFANTUM*

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Abstract

Impaired renal function is one of the main characteristics of dogs infected with *Leishmania infantum*. Early diagnosis of kidney injury is essential for improving patient's prognosis. This study aims to evaluate physiological biomarkers as predictors of kidney injury and prognostic markers. Medical records of fifty-nine dogs of different breed, age, and sex, naturally infected with *L. infantum,* were analyzed. Red blood cells, leucocytes, platelets count, hematocrit, total plasma proteins, plasma globulin, plasma albumin, serum creatinine, serum urea, serum phosphorus, serum symmetrical dimethyl arginine, urine analysis, urinary density, urinary protein creatinine ratio, urinary creatinine, urinary protein, and systemic blood pressure were examined in trial 0. Six months after trial 0, twenty-four dogs returned for clinical and laboratory examination. The second medical record analysis was identified as trial 1. The twenty-four dogs were examined using the same tests performed in trial 0. The physiological biomarker such as platelets and leukocyte count, hematocrit, serum phosphorus, urinary density, and systemic blood pressure, showed a significant correlation as prognostic and predictive factors of kidney injury in dogs. The platelet count was used as the physiological biomarker to show the value as a predictive factor and prognostic marker related to biomarkers of kidney injury in dogs naturally infected with *L. infantum*.

Key words: acute kidney injury, CanL, survival

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EVALUACIJA FIZIOLOŠKIH BIOMARKERA KAO MOGUĆIH PREDIKTIVNIH FAKTORA I PROGNOSTIČKIH MARKERA OŠTEĆENJA BUBREGA KOD PASA PRIRODNO INFICIRANIH SA LEISHMANIA INFANTUM

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Kratak sadržaj

Oštećena bubrežna funkcija je jedna od glavnih karakteristika pasa zaraženih sa Leishmania infantum. Rana dijagnostika oštećenja bubrega je od suštinskog značaja za bolju prognozu stanja pacijenta. Ova studija ima za cilj da proceni fiziološke biomarkere kao prediktore oštećenja bubrega i prognostičke markere. Izvršena je analiza medicinske dokumentacije za pedeset i devet pasa različitih rasa, starosti i pola, prirodno zaraženih sa L. infantum. Crvena krvna zrnca, leukociti, broj trombocita, hematokrit, ukupni proteini plazme, globulini u plazmi, albumin u plazmi, serumski kreatinin, serumska urea, serumski fosfor, serumski simetrični dimetil arginin, analiza urina, gustina urina, odnos urinarnog proteina i kreatinina, kreatinin u urinu, urinarni protein, i sistemski krvni pritisak ispitivani su kao parametri nultog ispitivanja. Šest meseci nakon nultog ispitivanja, dvadeset i četiri psa su se vratila na klinički i laboratorijski pregled. Podaci od drugog pregleda su označeni kao ispitivanje 1. Dvadeset četiri psa su pregledana korišćenjem istih testova izvršenih u ogledu „0“ (nultog ispitivanja). Fiziološki biomarkeri kao što su broj trombocita i leukocita, hematokrit, fosfor u serumu, gustina urina i sistemski krvni pritisak, pokazali su značajnu korelaciju kao prognostički i prediktivni faktori oštećenja bubrega kod pasa. Broj trombocita je korišćen kao fiziološki biomarker da pokaže vrednost kao prediktivni faktor i prognostički marker koji se odnosi na biomarkere oštećenja bubrega kod pasa prirodno inficiranih L. infantum.

Ključne reči: akutno oštećenje bubrega, lajšmanioza pasa, preživljavanje

INTRODUCTION

Visceral leishmaniasis (VL) is an infectious, systemic, and zoonotic disease caused by the protozoan *Leishmania infantum* and is transmitted by infected female sandflies, Lutzo*myia longipalpis,* and its main vector is in Brazil (Dantas-Torres and Brandão-Filho, 2006). It is estimated that there are millions of infected dogs in South America, especially in Brazil, where there are high rates of infection (Marcondes and Day, 2019)

Infected dogs can develop canine leishmaniosis (CanL), which can affect several organs and therefore exhibit several clinical characteristics, ranging from apparently healthy to severe illness and death, depending on the immune response triggered by the patient (Soares et al., 2005; Freitas et al., 2012)

Renal function impairment is quite frequent among the clinical changes observed in infected animals (Braga et al., 2015). The formation and deposition of immune complexes secondary to CanL can cause glomerulonephritis and tubulointerstitial lesions (De Oliveira Frazilio et al., 2018). Some authors believe that tubulointerstitial lesions occur secondary to glomerulopathy (Pardo-Marín et al., 2017; De Oliveira Frazilio et al., 2018). Renal proteinuria, which is a reflection of increased glomerular capillary permeability, is associated with the production of immune complexes and may be evidence of kidney injury (D'Amico and Bazzi, 2003; Soares et al., 2005; Freitas et al., 2012; Brown et al., 2013; IRIS 2023). Treatments for CanL in Brazil are based on miltefosine for leishmanicidal action, immunomodulatory medications such as domperidone, and immunotherapy, which consists of three double doses of the Leishtec® vaccine with intervals of twenty-one days between applications. Subsequently, a six-monthly booster should be administered with the application of a double dose. Immunotherapy was a good and efficient protocol for reducing clinical manifestations and controlling CanL relapses compared to other treatments (Santos et al., 2007; Ribeiro et al., 2013; Araujo and Gondim, 2020).

This study aims to evaluate physiological biomarkers as predictive and prognostic markers of the evolution of infection, renal injury, and renal failure in dogs naturally infected with *L. infantum*, assisting in the determination of treatment procedures, managing possible alterations identified, and in the prognosis of patients.

MATERIAL AND METHODS

For the retrospective data collection, no ethical approval was required as no identifying information was included, and no dogs were actively recruited.

Medical records of fifty-nine dogs of different breeds and ages naturally infected with *L. infantum* were examined. Out of the fifty-nine dogs studied, twenty-six were female dogs, while thirty-three were males. The dogs were classified and divided into stages as suggested by Solano-Gallego et al. (2011). The clinical staging of canine visceral leishmaniasis is classified in four stages based on serological status, clinical symptoms and laboratory findings. Dogs in Stage I - Negative to low positive antibody levels; Dogs with mild clinical symptoms such as peripheral lymphadenomegaly, or papular dermatitis. Usually, no clinicopathological abnormalities were observed. Normal renal profile was the following: creatinine <1.4 mg/dl; non-proteinuric: UPC < 0.5. Stage II - Low to high positive antibody levels; Dogs, which apart from the signs listed in stage I, may exhibit the following symptoms: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis / onychogryphosis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), anorexia, weight loss, fever, and epistaxis; Clinicopathological abnormalities such as mild non-regenerative anemia, hyperglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome. Substages: a) Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5 b) Creatinine <1.4 mg/dl; UPC = 0.5-1,0. Stage III - Medium to high positive antibody levels; Dogs, which apart from the symptoms listed in stages I and II, may show the signs originating from immune-complex lesions: vasculitis, arthritis, uveitis, and glomerulonephritis. Clinicopathological abnormalities listed in IRIS stage II of chronic kidney disease (CKD), creatinine 1.4-2,8 mg/dl, or stage I with UPC > 1. Stage IV - Medium to high positive antibody levels; Dogs with clinical symptoms listed in stage III. Pulmonary thromboembolism, or nephrotic syndrome (marked proteinuria UPC > 5) and final stage renal disease; Clinicopathological abnormalities listed in IRIS stage II CKD, stage III (creatinine 2,9-5mg/dl) and stage IV (creatinine >5 mg/dl).

The dogs from stage II and those undergoing treatment with immunotherapy (do Nascimento et al, 2018; Gouveia et al, 2021) as suggested by Brasileish (2019) were included. Medical records should also present parasitological examinations for *Leishmania sp.* identification by direct or molecular identification or titration above 1:160 in the indirect Fluorescent Antibody Test (IFAT) or sample value four times higher than the cut-off point in the Enzyme-Linked Immunosorbent Assay (ELISA) (Brasileish, 2019; Solano-Gallego et al., 2011; Laia et al., 2011) at trial 0 (T0). Eighteen (30.5%) dogs were classified as stage IIa (Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5), fifteen (25.4%) as stage IIb (Creatinine <1.4 mg/dl; UPC = 0.5-1), twenty-three (39%) in stage III, and three (5.1%) dogs were classified as stage IV (Solano-Gallego, et al., 2011).

Also, medical records should present complete blood count tests: red blood cell count (RBC), hematocrit (HC), leucocytes (LE) and platelets (PL), total plasma proteins (PROT), plasma globulin (GL), plasma albumin (AL), serum creatinine (CR), serum urea (UR), serum phosphorus (PH), serum symmetrical dimethyl arginine (SDMA), urine analysis, urinary density (UR. D), urinary protein creatinine ratio (UPC), urinary creatinine (CR. U) and urinary protein (UR PTN), all performed in the exact consultation, in the same laboratory. Systemic blood pressure (SBP) was measured using vascular doppler or through the oscillometer method with the animal positioned in lateral decubitus, in line with criteria for cuff size and minimum stress. Three to seven measurements were performed, and the first measurement was discarded to obtain an average of the other measurements, considering the patient's SBP value.

Animals that showed signs of dehydration, episodes of vomiting, diarrhea, inappetence, or lower urinary tract disease were excluded from the study. This first moment is identified as T0. Six months after T0, twenty-four dogs were returned for clinical and laboratory evaluation. The second medical record analysis was identified as trial 1 (T1). These twenty-four dogs were clinically healthy and evaluated through the same exams performed in the same laboratory in T0. Thirty-five dogs were not returned for evaluation or returned for more than six months and were therefore excluded from the study.

Statistical Analysis

Principal component analysis (multivariate analysis) was performed using numerical variables. This analysis evaluated all numerical variables, considering their correlation, and was performed using Spearman's correlation matrix. Moderate, strong, and robust correlations were reported, with confidence intervals above 95%. Statistical analyses were performed using the R software version ("The R Project for Statistical Computing," 2019).

RESULTS

T0

The distribution of the dogs evaluated in this study is shown in Figure 1. The arrows facing the same direction indicate biomarkers that are positively correlated with each other. The points in the two quadrants on the right showed higher values for CR, UR, SDMA, PH, UPC, and UR. PTN. In these

quadrants there were higher concentration of dogs classified in stages III and IV, whereas those in the two left quadrants, mostly dogs classified in stages IIa and IIb, had higher values mainly for CR. U, HC, AL, and UR. D. The points in the two upper quadrants had higher PL counts. On the other hand, the points in the two lower quadrants had higher values, mainly for PROT and GL. There was a relative separation in the group IIa, with animals concentrated on the left side of the graph, and group IV, with animals focused on the right side. The chart shows the dispersion and evolution of dogs from T0 to T1. The dogs classified as stage IIa or IIb were in the left quadrant. The dogs in stages III and IV were in the right quadrant, with high levels of CR, UR, SDMA, PH, and UPC, demonstrating that patients classified in the final stages have a greater impairment of the glomerular filtration rate.

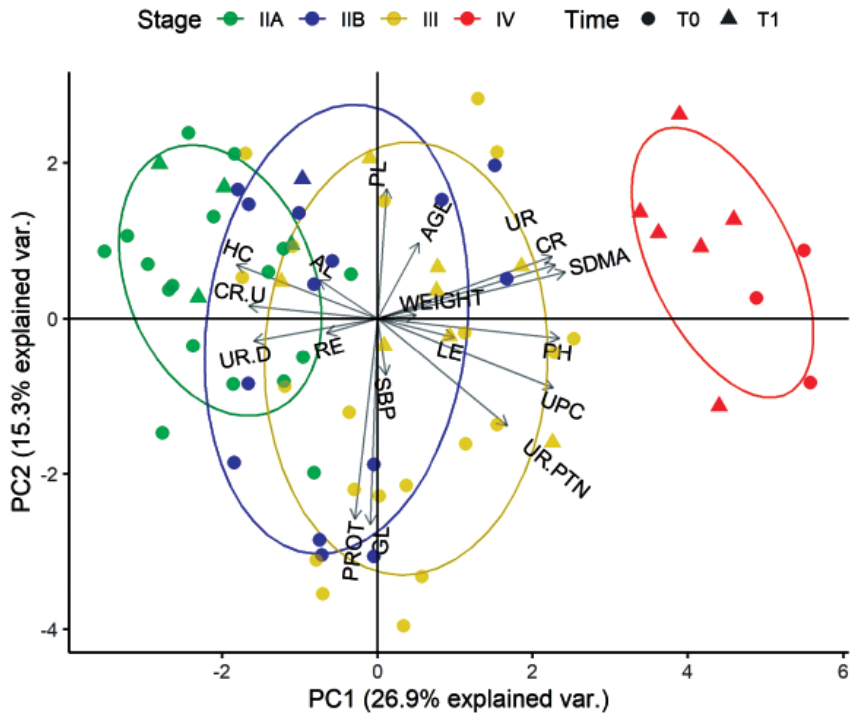

Figure 1: Dispersion of dogs considering the staging and the values obtained in the analytes studied at T0 and T1. The points in the two quadrants on the right showed higher values for CR, UR, SDMA, PH, UPC, and UR. PTN, whereas those in the two left quadrants showed higher values mainly for CR, UR, HC, AL, and UR. D. The points in the two upper quadrants had higher PL counts, while the points in the two lower quadrants had higher values, mainly for PROT and GL.

Anemia was found in twenty-one (35%) dogs out of fifty-nine analyzed in the study. Two (11%) of the dogs were classified as stage IIa, five (33%) as stage IIb, 11 (47%) in stage III, and three (100%) in stage IV present anemia.

Thrombocytopenia was present in seventeen (28%) dogs out of fifty-nine evaluated in the study. Three (16%) of the dogs classified as stage IIa, four (26%) in stage IIb, seven (30%) in stage III, and three (100%) in stage IV had thrombocytopenia.

Out of the thirty-three dogs at T0, classified by Solano-Gallego et al. (2011) IIa and IIb stages evaluated in this study, in thirteen (39%), UPC was the first biomarker to assess kidney injury to have the values higher than reference values. SDMA was the first evaluated biomarker with values higher than reference values in two dogs (6%). SDMA and UPC levels increased simultaneously in two dogs (6 %) classified by Solano-Gallego et al. (2011) IIa and IIb stages.

SDMA had the values above the reference (> 18 µg/dL IRIS, 2023) in one (5%) dog classified as stage IIa and in four (26%) dogs classified as stage IIb. In stage III, there was an increase in SDMA in eight (34%) dogs, and in stage IV in three (100%) dogs.

Analyzing correlations at T0, they were found to be moderate with values varying between 0.4 and 0.69, and strong between 0.70 and 0.89. SDMA had a moderate positive correlation with UR and CREAT and a moderate negative correlation with HC. UR levels had a strong positive correlation with serum CR levels and a moderate positive correlation with PH levels. PH showed a reasonable positive correlation with UPC and a moderate negative correlation with RBC and HC. PROT levels showed a moderate negative correlation with the PL count. AL levels had a moderate positive correlation with HC and RBC, which was negatively correlated with UPC and UR. PTN levels. UR. D showed a moderate correlation with CR.U.

Comparisons between T0 X T1

The correlation of biomarkers evaluated at T0 and T1 is shown in Table 1. When correlations were evaluated at T0 and T1, PL (T0) had a negative correlation with UPC and UR. PTN levels at T1. There was no correlation between PL and UPC in T0. The UR. D (T0) and UPC (T1) showed a strong negative correlation. LE (T0) had a moderate correlation with UR (T1). PH (T0) had a moderate correlation with UR. PTN.

In the analysis of the correlation between the variables in T0 and the variables of dogs with CR lower than 1.4 mg/L in T1 is shown in Table 2. UR D (T0) had a negative correlation with the UPC (T1) and the GL (T1). SDMA

(T0) was correlated with CR. U level (T1). PL (T0) correlated with UR and PH levels (T1). UR. PTN (T0) levels are associated with PROT levels (T1). PH and SBP (T0) were correlated with LE (T1). It was not possible to perform the analysis for animals with serum CR equal to or greater than 1.4 mg/L, as due to an insufficient number of dogs, it is not possible to ger a basis for interpretable analysis.

Table 1: Correlations between the biomarkers studied at T0 and T1 that were statistically significant.

BIOMARKER	TRIAL 0	BIOMARKER	TRIAL 1	CORRELATION	P
CR.U	T0	UR.D	T1	0.73	0.003
GL	T0	LE	T1	0.48	0.019
CR.U	T0	UR. PTN	T1	-0.71	0.023
PL	T0	UPC	T1	-0.69	0.023
PL	T0	UR. PTN	T1	-0.69	0.026
PROT	T0	GL	T1	0.45	0.027
UR. D	T0	UPC	T1	-0.69	0.027
SDMA	T0	CR. U	T1	0.68	0.029
CR. U	T0	UPC	T1	-0.68	0.03
LE	T0	UR	T1	-0.44	0.031
PROT	T0	LE	T1	0.43	0.031
PL	T0	LE	T1	-0.43	0.036
PH	T0	UR. PTN	T1	0.66	0.036
HC	T0	PTOT	T1	-0.42	0.043
UR. PTN	T0	UPC	T1	0.65	0.043
PL	T0	PH	T1	-0.41	0.048
SBP	T0	LE	T1	0.41	0.049

Hematocrit (HC), leucocytes (LE) and platelets (PL), total plasma proteins (PROT), serum globulin (GL), serum urea (UR), serum phosphorus (PH), serum symmetrical dimethyl arginine (SDMA), urine analysis, urinary density (UR. D), urinary protein creatinine ratio (UPC), urinary creatinine (CR. U), urinary protein (UR PTN) and systemic blood pressure (SBP).

Table 2: Correlation of the variables in T0 together with the variables of dogs that present creatinine lower than 1.4 mg/L at T1 that presented statistically significant.

BIOMARKER	TRIAL 0	BIOMARKER	TRIAL 1	CORRELATION	P
UR. D	T0	UPC	T1	-0.92	0.001
SDMA	T0	CR. U	T1	0.92	0.001
PL	T0	UR	T1	-0.59	0.013
UR. D	T0	GL	T1	0.57	0.016
SBP	T0	LE	T1	0.56	0.018
PL	T0	PH	T1	-0.55	0.022
PH	T0	LE	T1	0.53	0.03
UR. PTN	T0	PROT	T1	0.5	0.042

Hematocrit (HC), leucocytes (LE) and platelets (PL), total plasma proteins (PROT), serum globulin (GL), serum urea (UR), serum phosphorus (PH), serum symmetrical dimethyl arginine (SDMA), urine analysis, urinary density (UR. D), urinary protein creatinine ratio (UPC), urinary creatinine (CR. U) and urinary protein (UR PTN) and Systemic blood pressure (SBP).

DISCUSSION

The importance of evaluating coinfections in dogs infected with *L. infantum* is well known. Infections with *Babesia canis*, *Anaplasma platys*, *Hepatozoon canis* and *Ehrlichia canis* cause hematological alterations such as anemia, pancytopenia, and thrombocytopenia (Thrall, 2007; Vilela et al., 2013), which may change the values of laboratory tests in the studied dogs. The lack of this evaluation presents a limitation of this study.

Changes in the erythrogram of dogs infected with *L. infantum* were observed in this study, in the form of mild to moderate anemia and thrombocytopenia. However, the pathogenesis of anemia in CanL includes additional mechanisms such as reduced erythropoietin synthesis due to renal failure (Paltrinieri et al., 2016). Anemia may also result from the parasite's invasion of the bone marrow, which induces inflammation that may contribute to a decrease in erythrocyte production (Da Costa-Val et al., 2007). Anemia is usually present in normocytic and normochromic forms with a non-regenerative character (Freitas et al., 2012). Thrombocytopenia is a common finding in dogs with CanL and occurs due to vasculitis caused by circulating immune complexes,

thrombocytopoiesis disorders, and PL destruction (Solano-Gallego L., et al., 2009; De Carvalho Nicolato et al., 2013).

Hyperproteinemia is caused by hyperglobulinemia, which is associated with the activation of B lymphocytes and high antibody production (Freitas et al., 2012; Paltrinieri et al., 2016; Maia and Campino, 2018). A decrease in the production or renal loss of AL was also observed in this study, but the increase in GL levels was very expressive, causing the levels of PROT to increase significantly during CanL (Giunchetti et al., 2008; Solano-Gallego, et al., 2009). As for urinalysis, in the present study, proteinuria was the most frequent alteration in animals with CanL to both mild or severe degrees, as described by Amusategui et al. (2003) and Bonfanti and Zatelli (2004).

Glomerulonephritis, tubulointerstitial nephritis, and nephropathy are common in CanL (De Oliveira Frazilio et al., 2018). Initially, glomerulonephritis may manifest as asymptomatic proteinuria (Koutinas and Koutinas, 2014; Paltrinieri et al., 2016), but with its progression, excretion dysfunction can occur in the presence of an increase or decrease in the glomerular filtration rate (GFR) and systemic hypertension (Plevraki et al., 2006; Cortadellas et al., 2008). An increase in GFR associated with hypertension can amplify glomerulopathy, resulting in the progression of CKD (Koutinas and Koutinas, 2014). End-stage CKD is a severe manifestation of disease progression and the leading cause of death in CanL. The two main parameters used to classify the degree of kidney disease in CanL, according to (Solano-Gallego et al., 2011; Laia et al., 2011), are UPC, used as a marker of glomerular pathology, and serum CR level, used as a marker of renal excretion (Torrent et al., 2018).

In 2019, IRIS included SDMA levels in the CKD staging of chronic kidney disease. SDMA is methylated arginine produced by cellular catabolism correlated with CREAT and GFR (Nabity et al., 2015). Several studies have demonstrated that SDMA levels are elevated in dogs with CKD (Hall et al., 2014; Nabity et al., 2015). Studies have suggested that SDMA levels also increase earlier than serum CR levels (Hall et al., 2014; Nabity et al., 2015) because its production is not influenced by the loss of muscle mass. SDMA showed a moderate correlation with UR and PH in T0. An increase in SDMA, UR, PH, and CR and a reduction in UR is expected as there is a worsening of renal excretion and a decrease in TGF.

There is a paucity of data on the behavior of SDMA in dogs naturally infected with *Leishmania* sp., with only four studies reported to date (Pardo-Marín et al., 2017; De Oliveira Frazilio et al., 2018; Torrent et al., 2018; Giapitzoglou et al., 2020). The serum concentration of SDMA is elevated mainly in azotemic dogs with severe proteinuria and decreased UR. D (Giapitzoglou et

al., 2020). SDMA correlated with CR and UR. D, a result like that obtained by (Giapitzoglou et al., 2020). A study carried out by (Torrent et al., 2018) found different results, without 0observing a correlation between SDMA and CR.

SDMA was also inversely correlated with the red RBC and HC. Kidneys perform numerous metabolic functions, including their contribution to erythropoiesis. Several factors contribute to anemia in patients with reduced GFR and CanL. The reduction of erythropoietin of renal origin is an important cause, but other mechanisms, such as anemia of inflammatory origin, the influence of uremic toxins on the survival time of the erythroid lineage, cofactor deficiency, blood loss due to various bleeding events, and parasitic destruction (Babitt and Lin, 2012; Fiocchi et al., 2017; Lippi et al., 2021).

There was a significant and progressive increase in the number of dogs with SDMA levels above the reference values (IRIS, 2023) as the stage progressed. The expected result of disease progression between stages demonstrated that SDMA could be used to classify CanL severity (Giapitzoglou et al., 2020). A different result was found by Torrent et al. (2018), who found no significant difference in the comparison between the stages (Solano-Gallego et al., 2011).

In this study, two dogs classified in stage IIa (non-proteinuric and non-azotemic) with elevated SDMA levels were observed. (Cortadellas et al., 2008) pointed out that dogs with CanL and UPC levels between 0.2 and 0.5 may have a decrease in GFR. Dogs with CanL may have some renal perfusion impairment secondary to hypovolemia, which could cause an increase in SDMA concentration without an association with proteinuria or renal azotemia. Torrent et al. (2018) suggested that dogs with CanL and changes in SDMA concentration without proteinuria should be carefully examined for pre-renal causes of impaired renal perfusion.

UPC was shown to be an earlier diagnostic sign of acute kidney injury (AKI) concerning SDMA in dogs naturally infected with *L. infantum* observed in this study, which is in line with other studies (Pardo-Marín et al., 2017; Torrent et al., 2018; Giapitzoglou et al., 2020). Glomerulonephritis is the main pathological event of nephropathy in CanL, and proteinuria is the primary laboratory alteration. However, glomerular pathology can cause a reduction in GFR and an increase in the serum concentrations of SDMA and CR (Koutinas and Koutinas, 2014; Paltrinieri et al., 2016)

When evaluated as prognostic markers and predictors of kidney injury and kidney failure, the UPC and SDMA levels did not show a statistically significant correlation. Pardo-Marin et al. (2017) identified a reduction in the concentration of UPC, however, they did not observe a decrease in the SDMA levels in dogs after leishmanicidal treatment. In this study, we observed a cor-

relation between SDMA (T0) and CR. U (T1) levels; however, as the dogs examined in this study underwent a treatment with immunotherapy, an improvement in GFR may have occurred after the reduction of the injury caused by CanL. Gouveia et al, 2021 found that immunotherapy with vaccines was the most effective treatment for negative serology in the ELISA and IFAT tests after the treatments compared to other treatments in the study. The decrease in the result in the serology may suggest a reduction in the production of antigen-antibody complexes influencing the GFR.

An increase in PROT and GL levels, associated with a decrease in AL levels, has been observed in several studies (Artan et al., 2006; Sales et al., 2017). This increase in PROT and the presence of hyperglobulinemia are considered to be among the most common alterations in CanL and may be associated with increased levels of anti-*Leishmania* antibodies, especially in severe stages of the disease (Castro et al., 2012; Freitas et al., 2012). However, hypoalbuminemia observed in animals may be due to the migration of albumin to the extravascular environment, with the formation of edema, a widespread clinical change in CanL (Silva, 2007; Freitas et al., 2012) and/or associated with an inflammatory process and/or albuminuria (Pierantozzi et al., 2013; Proverbio et al., 2016). Given the processes that occur during CanL due to the association of GL with the stimulation of B lymphocytes (Vieira et al., 2021), we can explain why the serum concentration of GL and PROT (T0) had a significant correlation with LE (T1). AL levels did not significantly correlate with any of the biomarkers.

The development of glomerulopathies not only leads to complications resulting from the accumulation of uremic toxins and fluid and electrolyte imbalances, but also causes systemic arterial hypertension, aggravating the clinical picture of patients and possibly compromising other organs, such as the heart (Schiffrin et al., 2007). In this study, seventeen (28%) dogs were identified with SBP above 160 mmHg. Out of these, seven (41%) were non-azotemic and non-proteinuric. Cortadellas et al. (2008) observed that 49.5% (52 / 105) of the dogs had some degree of renal disease, and 61.5% (32 / 52) of these dogs were diagnosed with systemic hypertension (SH). Moreover, SH also was diagnosed in 2% of dogs without renal disease. Braga et al. (2015) found that all dogs with hypertension had histopathological and laboratory evidence of glomerular disease. However, there was no statistically significant correlation between elevated BP and the severity of glomerular lesions.

This study found that SBP (T0) correlated with LE count (T1). CanL glomerulonephritis can form large antigen-antibody complexes, inciting inflammation and overloading of glomerular capillaries, resulting in obstruction that can further elevate glomerular pressure (Harrison et al., 2012). There is also

evidence that inflammatory cells accumulate in the perivascular region of the kidney (Theuer et al., 2002) also contributing to hypertension.

Unlike SBP, data in the literature cite the destruction of LE in uremic patients (Minnaganti and Cunha, 2001; Cohen and Hörl, 2012; Pahl et al., 2010). In this study, predictive factors of LE (T0) and HC (T1) concerning UR were observed through an inverse correlation. Uremia is associated with hematological abnormalities such as hemostatic, granulocytic, lymphocytic, and PL disorders, mainly caused by chemotaxis, phagocytosis, and oxidative abnormalities. Some diseases related to antigen presentation have also been reported in uremic patients (Minnagantiand and Cunha, 2001; Cohen and Hörl, 2012) demonstrated that patients with renal failure had impaired body defense. Pahl et al. (2010) reported that the number of B lymphocytes and their ability to produce antibodies was reduced in patients with uremia. A study conducted on dogs infected with *Leishmania sp.*, showed more intense hematological alterations, such as profound anemia, thrombocytopenia, and leukopenia, characterizing pancytopenia associated with bone marrow hypoplasia or aplasia as a result of the invasion of the microorganism and immune-mediated processes (Paltrinieri et al., 2016). Hyperplasia and peripheral cytopenia can partially be attributed to the increased destruction of mature blood cells in the periphery. There are also morphologic features indicative of differentiation blockage and dyserythropoietic changes in the erythroid precursors (Poulaki et al., 2021)

HC (T0) also showed an inverse correlation with serum PROT (T1). Thus, the presence of leukopenia and anemia may indicate an initial process of immunosuppression and an increase in parasite load, which may lead to a change in hyperglobulinemia, an initial process of renal injury, and disease progression.

PH concentrations were also moderately correlated with UR and PTN levels. A study demonstrated an essential relationship between PH and proteinuria. When analyzing the renal protective response, it was found that for a reduction in proteinuria, a reduction in protein intake was not necessary, but a reduction in PH, either in its urinary excretion or serum concentration. Patients with low PH levels had the most significant decrease in proteinuria, regardless of UR excretion (a factor used to estimate protein intake) (Di Iorio et al., 2013).

Based on all findings and discussion it can be concluded that the most associated parameters are PL and SBP. In order to interpret the results and analyze the predictive factors and prognostic markers, we analyzed Figure 1 and isolated four patients who presented a negative evolution, starting from classification in stage IIa at T0 to stage IV at T1. Two patients developed thrombo-

cytopenia and systemic arterial hypertension at T0. Neither of the patients had proteinuria, azotemia, or hyperglobulinemia and had SDMA < 14μg/dL. The authors pointed out the importance of evaluating systemic hypertension and thrombocytopenia in dogs with *L. infantum* infection. The other two patients exhibited no changes in laboratory test results at T0. New studies should be carried out in order to evaluate PL and SBP as predictive factors and prognostic markers.

CONCLUSIONS

PL was the primary physiological biomarker to demonstrate value as a predictive factor and prognostic marker being related to biomarkers of kidney injury as PH and UPC. It has been shown that SBP, LE, HC, PH and UR. D are predictive or prognostic markers in dogs infected with *L. infantum.* There was no advantage of SDMA over UPC for assessing kidney injury in dogs infected with *L. infantum* at the time of the study. SDMA and UPC didn´t show significance as predictive or prognostic markers in dogs infected with *L. infantum*. It is concluded that the evaluation of thrombocytopenia is important in the evaluation of CanL, and its reversal corresponds to an improvement in the prognosis.

Author's Contributions

FSM and APCV: data collection, analysis and experimental design. They conducted the gathered the necessary data and performed statistical analysis on the results; assisted in data interpretation and manuscript preparation. They contributed equally to the interpretation of the experimental results, helped in drafting the manuscript, and critically reviewed the content for accuracy and clarity. JCCV and VMR contributed to the theoretical framework and literature review.

Competing interest

The author(s) declare that they have no competing interests.

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INFECTIOUS STOMATITIS IN CAPTIVE *SALVATOR MERIANAE* LIZARDS

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Abstract

Infectious stomatitis is a common ailment in captive reptiles, it arises from a combination of predisposing factors such as poor nutritional conditions, habitat issues or stress, where normal oral flora microorganisms act as pathogens, facilitating the disease development. This study aims to describe infectious stomatitis in a captive population of adult *Salvator merianae* lizards and to propose prophylactic measures for their maintenance in captivity. Within a population of 57 animals, a morbidity rate of 19.2% and a mortality rate of 7% were estimated. Microbiological analysis of oral mucosa revealed *Pseudomonas aeruginosa* susceptible to ceftazidime, ciprofloxacin, gentamicin, and amikacin. Symptoms ranged from asymptomatic to animals with mild oral cavity lesions or severe stomatitis, with some cases exhibiting respiratory complications. Histopathological examination of lung samples was consistent with caseous pneumonia. Intramuscular ceftazidime treatment and oral disinfection yielded excellent results for lizards with stomatitis, although a favorable response was not observed in animals with pneumonia.

Key words: Black and white tegu, caseous pneumonia, oral diseases, *Pseudomonas aeruginosa*, reptiles

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INFEKTIVNI STOMATITIS KOD GUŠTERA VRSTE *SALVATOR MERIANAE* KOJI SE DRŽE U ZATOČENIŠTVU

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Kratak sadržaj

Infektivni stomatitis je česta bolest kod gmizavaca koji se drže u zatočeništvu. Uzrokuje ga kombinacija predisponirajućih faktora kao što su loša ishrana, problemi sa staništem ili stres, gde normalni mikroorganizmi oralne flore deluju kao patogeni, što potpomaže razvoj bolesti. Ova studija ima za cilj da opiše infektivni stomatitis u populaciji odraslih guštera Salvator merianae koji se drže u zatočeništvu i da izloži predloge profilaktičkih mera za njihovo držanje u zatočeništvu. U okviru populacije od 57 životinja utvrđena je stopa morbiditeta od 19,2% i mortaliteta od 7%. Mikrobiološka analiza oralne sluzokože pokazala je prisustvo *Pseudomonas aeruginosa* koji je osetljiv na ceftazidim, ciprofloksacin, gentamicin i amikacin. Simptomi su bili raznoliki - od asimptomatskih životinja do onih koje su ispoljile simptome teškog stomatitisa, blage lezije usne duplje, a neke su imale i respiratorne komplikacije. Histopatološki pregled uzoraka pluća ukazao je na kazeoznu pneumoniju. Intramuskularni tretman ceftazidimom i oralna dezinfekcija dali su odlične rezultate za guštere sa stomatitisom. Međutim, nije primećen povoljan odgovor kod životinja sa pneumonijom.

Ključne reči: Crni i beli tegu, kazeozna pneumonija, oralne bolesti, *Pseudomonas aeruginosa*, reptili

INTRODUCTION

Adequate zootechnical management stands out as the most crucial factor for maintaining healthy reptiles bred in captivity. Inappropriate breeding conditions, such as overcrowding, nutritional deficiencies, infections, and parasitosis lead to stress-induced immunosuppression (Cobos and Ribas 1987; Meredith and Redrobe, 2012; Zhou et al., 2020; Tian et al., 2022). Numerous microorganisms constitute oral microbiota in reptiles. Nevertheless, disrup-

tions in the homeostasis of the immune response caused by the captive environment transform the commensal oral microbiota into opportunistic pathogens (Grego et al., 2017; Vega-Manriquez et al., 2018).

Infectious stomatitis, or "mouth rots," is a common oral mucosa infection in reptiles bred in captivity. While this disease has been predominantly described in snakes (Peñuela Gomez and Brieva Rico, 2007; Rojas-Sereno et al., 2015; Martins et al., 2021), it also affects turtles and some lizard species (Herrera Ramírez, 2008; Pereira et al., 2021). Symptoms of this pathology include sialorrhea, petechiae, plaques around the lips and mouth, facial malformations, gingival abscesses, and teeth loss. In cases that are not promptly controlled, the exudate from ulcerative stomatitis may be swallowed or aspirated, leading to gastroenteritis or bacterial pneumonia. In severe cases, the pathogen may also enter the general circulation, causing septicemia and death (Cobos and Ribas 1987; Mader, 2006; Meredith and Redrobe, 2012; Rodríguez Molano, 2015; Pereira et al., 2021; Rojas-Sereno et al., 2015; Doneley et al., 2018).

In saurians, pathological processes tend to be multifactorial, slow-progressing, and challenging to diagnose due to their inherent resistance and ability to mask symptoms. For this reason, in most cases, diseases are detected at advanced stages, which complicates the success of treatment and, in the case of captive populations, epidemiological control (Mader, 2006; Meredith and Redrobe, 2012).

This study provides a detailed report on the clinical manifestations, lesions, and treatment in a captive population of *Salvator merianae* lizards with infectious stomatitis.

MATERIALS AND METHODS

Animals

The study included 57 adult individuals from the *Salvator merianae* lizard breeding facility at the Facultad de Agronomía, Zootecnia y Veterinaria of the Universidad Nacional de Tucumán, province of Tucumán, Argentina (26° 51'S and 65° 17'W). The animals were housed in open-air enclosures with masonry fences, equipped with shelters containing dry grass and shade. For the welfare of adult *Salvator merianae* individuals, the minimum living space of 2 m^2, was provided (Manes, 2016). Ad-libitum feeding consisted of a diet specifically designed for this captive-bred species (Vega Parry and Manes, 2000). For individual health monitoring, radiofrequency identification devices (micro transponder ID-100, Trovan Electronic Identification, Rosenbusch, Buenos Aires, Argentina) were used.

All experiments, including all animal handling protocols, were carried out in accordance with the Principles of Laboratory Animal Care (National Institutes of Health, publication N° 85- 23, revised 1985), as well as specific national laws. All experiments were carried out and approved by the Ethics Committee of Consejo de Investigaciones de Universidad Nacional de Tucumán (CIUNT).

Characterization of pathogens

The samples from the buccal and gingival mucosa of animals exhibiting signs of advanced stomatitis were collected using swabs and transported and preserved in Stuart medium. Bacteriological culture and antibiogram testing were conducted using the agar diffusion method (Kirby-Bauer test).

Histopathology

Histopathological analysis of lung biopsies from recently deceased animals was performed using standard procedures, including fixation with 10% neutral buffered formalin, embedding, sectioning, and hematoxylin and eosin staining (Suvarna et al., 2018).

RESULTS

Epidemiology and clinical signs

The disease affected both males and females. Out of a total number of 57 adult animals that were examined, 4 exhibited symptoms of advanced stomatitis, 7 showed signs of mild stomatitis, and another 4 were cases with respiratory and/or systemic complications. The remaining 42 animals did not show any lesions in the oral mucosa indicative of clinical stomatitis. As population indicators, the morbidity rate of stomatitis with clinical signs was estimated at 19.2%, with a mortality rate of 7%.

The 4 individuals with the signs of advanced stomatitis had petechiae and ecchymosis in the oral mucosa, periodontitis, they experienced loss of dental pieces, and granulomatous plaques in the oral and lingual mucosa. In some cases, these signs were accompanied by oral, ocular, or nasal discharge (Figure 1A).

The seven animals that manifested mild signs of stomatitis, had inflammation of the oral and gingival mucosa, but without hemorrhagic lesions or any type of plaques.

The 4 animals with severe stomatitis and caseous necrosis in the mouth and tongue died due to respiratory and systemic complications. Only two of them showed evident signs of respiratory distress with dyspnea, white foamy expectorations, and loss of appetite. The other 2 did not show evident signs of respiratory complications, and the diagnosis was confirmed through post-mortem lesions (Figure 1B).

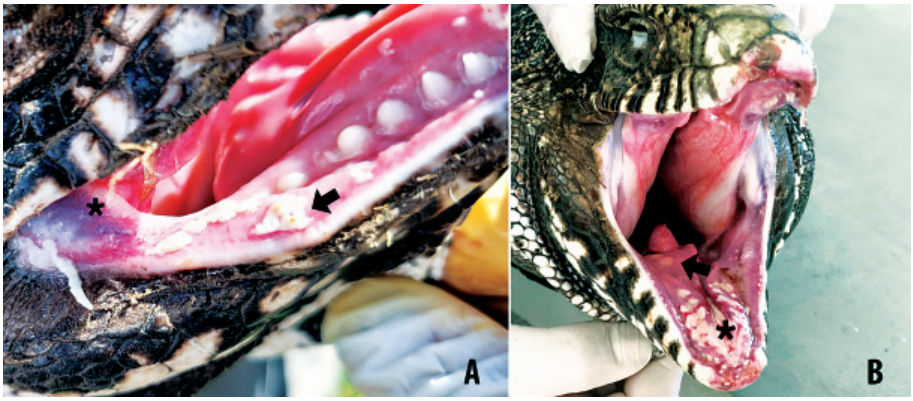

Figure 1. A. Adult female *S. merianae* lizard with stomatitis; arrow indicates granulomatous plaque on oral mucosa, asterisk indicates inflamed, congested, and ecchymotic area at oral commissure. B. Adult male *S. merianae* lizard with severe stomatitis; asterisk indicates apical region of the tongue and oral mucosa with caseous necrosis, arrow indicates granulomatous plaque on the body of the tongue.

Macroscopic findings

The necropsy of the recently deceased lizards revealed that the most affected organs were the lungs, although some macroscopic lesions were also found in the mouth, the fat bodies, and the liver. The fat bodies were notably congested and friable, with hemorrhagic lesions. Additionally, the friable liver had changed in its color. The lungs were the most affected organs, significantly hyperemic, and hemorrhagic with numerous pinpoint granulomatous plaques similar to those found in the oral cavity (Figure 2).

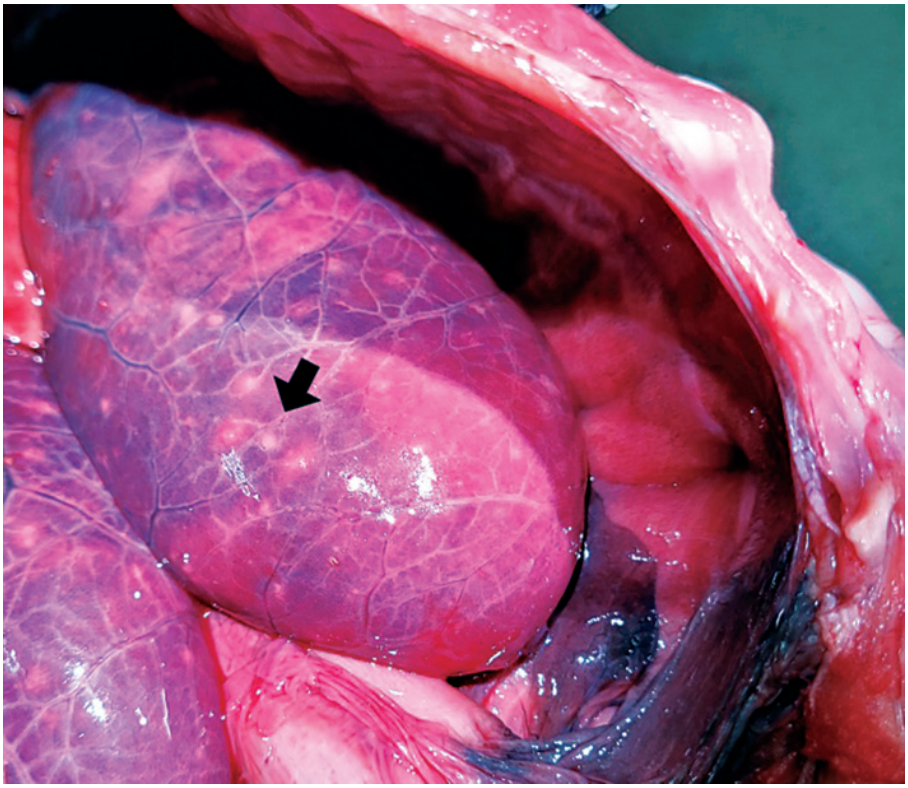

Figure 2. Lungs with numerous small granulomatous lesions (arrow).

Histopathology findings

In the submesothelial layer of the visceral serous tunic and the stroma of type III trabeculae in both lungs, inflammatory hyperplasia, characterized by the profusion of collagen fibers, was evident. Moreover, in the lumen of the pulmonary venules, there was a notable abundance of lymphocytes (Figure 3A). Necrotic lesions with infiltration of the pulmonary parenchyma and eosinophilic areas centrally located to the granulomatous lesion, composed of distinctive amorphous tissue of caseous necrosis, were also prominent (Figures 3B and 3C).

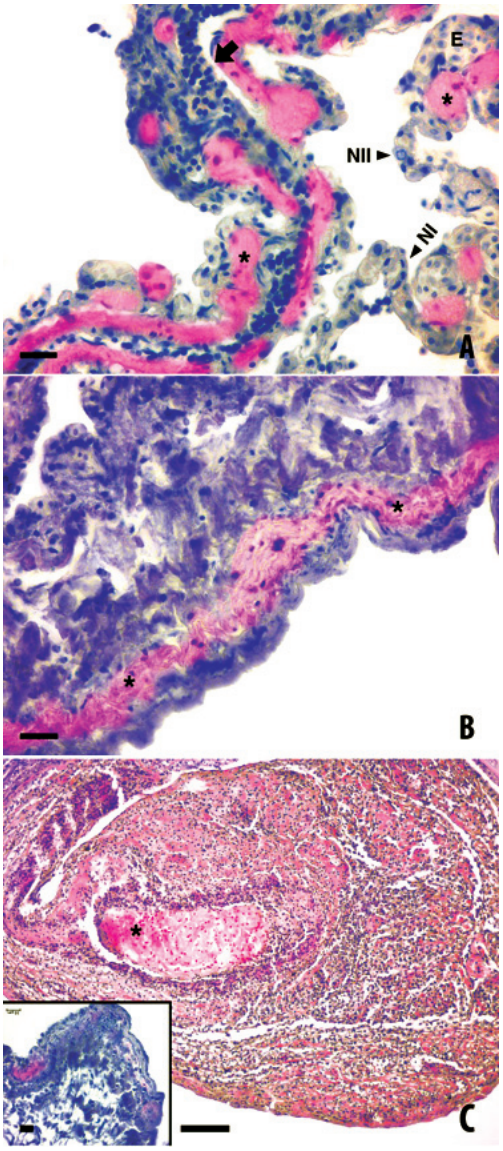

Figure 3. A. Detailed view of foveolar epithelium with marked hypertrophy of type III trabeculae (asterisks). Note the abundance of lymphocytes (arrow). NI, type I pneumocytes; NII, type II pneumocytes; E, erythrocytes. Scale bar 10 μm. B. Detailed view of visceral serous layer of the lung. Note the abundance of collagen fibers in the submesothelial layer (asterisks). Scale bar 10 μm. C. Lung section with granulomatous lesion (asterisk). Scale bar 50 μm. The insert shows characteristic necrotic tissue with absence of cellular boundaries and pyknotic nuclei. Scale bar 10 μm.

Culturing and antibiogram

Bacteriological culture of the oral mucosa of animals with the clinical disease revealed few Gram-positive cocci and few Gram-negative bacilli, as well as polymorphic nuclear cells. *Pseudomonas aeruginosa*, sensitive to antibiotics such as ceftazidime, ciprofloxacin, gentamicin, and amikacin, was isolated.

Treatment and evolution of animals with clinical signs

Based on the antibiogram results, intrinsic characteristics of each antibiotic (therapeutic margin, toxicity, duration of action, ease of application), and cost analysis, ceftazidime antibiotic therapy was chosen.

Animals with the signs of stomatitis with evident oral mucosa plaques and lesions underwent the following treatment: cleaning of wounds with 10 vol. hydrogen peroxide or 0.05% chlorhexidine and manual removal of oral plaques. Simultaneously, they were supplemented with 5000 IU of oral vitamin A once a week.

Depending on the characteristics of each patient and the severity of the clinical picture, between 3 and 6 applications of intramuscular ceftazidime at 30 mg/kg were performed every 72 hours until the reversal of symptoms.

In cases of mild or advanced stomatitis without respiratory complications, the treatment was highly effective, and the patient's evolution was favorable (Figure 4). However, in animals with clinical signs of pneumonia, the treatment was not effective.

Figure 4. Evolution of a stomatitis case: A and B. Initial state of oral wounds, left and right profiles, respectively. C. Oral wounds at 2 weeks after the start of treatment. Arrows indicate granulomatous plaques at both commissures, asterisk indicates petechiae. D. Resolution of oral wounds and patient's discharge 6 weeks after treatment initiation.

Population Management Measures

For metaphylactic treatment, the entire breeding stock received a single dose of 30 mg/kg of intramuscular ceftazidime. Weekly controls of the oral mucosa and body condition of 10 randomly selected animals were conducted for a month. When an individual showed any signs or suspected symptoms of stomatitis, it was isolated to start antibiotic treatment, vitamin A supplementation, and cleaning and disinfecting of the oral mucosa.

After a month, and in order to avoid stress from manipulation, inspections consisted of visual examination of animals in the corral, and only if suspicious, the animal was restrained for mucous membrane inspection. An animal was considered suspicious if it exhibited at least one of the following symptoms:

oral, ocular, or nasal discharge of any type, weight loss of body condition, or dehydration.

Sick or suspected animals were separated from the rest in an isolation area with the same shade, shelter, water, and ration conditions as the rest of the enclosures. Feeders, drinkers, and shelters throughout the breeding facility were disinfected with 0.1% sodium hypochlorite, and the substrate in all shelters was renewed.

DISCUSSION

The oral and intestinal microbiota varies significantly among different groups of reptiles and is influenced by their habitat, physiology, and nutrition. Generally, the oral flora in saurians is primarily composed of Gram-negative bacteria, such as *Escherichia coli, Salmonella, Pseudomonas, Proteus,* and *Aeromonas* (Meredith and Redrobe, 2012; Tian et al., 2020, 2022). These bacteria can be isolated from both healthy and diseased animals. It is known that healthy reptiles can transmit *Salmonella* or *P. aeruginosa*, representing a major zoonotic risk associated with keeping reptiles (Meredith and Redrobe, 2012; Martins et al., 2021). This is the first study reporting *P. aeruginosa* as a potential opportunistic pathogen causing infectious stomatitis in *S. merianae.*

The clinical signs observed in our animals coincided with those reported for stomatitis in other captive reptiles (Cobos and Ribas 1987; Mader, 2006; Meredith and Redrobe, 2012; Rojas-Sereno, 2015; Hedley, 2016; Doneley et al., 2018; Pereira, 2021). The lesions found in the oral cavity of diseased animals were similar to those described for *Boa constrictor amarali* with caseous stomatitis caused by *P. aeruginosa* (Martins et al., 2021). In reptiles, pneumonia signs often appear late, when the infection is chronic with significant respiratory involvement (Mader, 2006; Rodríguez Molano, 2015). In most cases observed in this study, the condition was limited to oral infection. However, in animals with pneumonia, macroscopic and histopathological lesions were consistent with chronic inflammatory processes, characterized by a marked increase in collagen fibers and notable lymphocytic infiltration. Timely treatment with intramuscular ceftazidime every 72 hours and oral antiseptics proved to be the appropriate combination for treating stomatitis in *S. merianae*. The combination of clinical examination, oral culture and antibiogram, histopathology, and favorable response to treatment, enabled us to confirm the diagnosis without the need for more expensive complementary tests.

The incidence of bacterial diseases in captive lizards is often associated with a compromised immune system resulting from overcrowding, trauma, nutritional deficiencies, infections, and excessively low temperatures that can

predispose normal microbial flora to act as opportunistic pathogens (Mader, 2006; Hedley, 2016; Van Zanten and Simpson, 2021). Regarding predisposing factors, overcrowding and poor management as promoters of stomatitis were ruled out. Indeed, we have optimized the zootechnical management of captive *S. merianae*, as well as the breeding conditions in which these lizards are kept and reproduced. This includes spacious enclosures with over 2 m^2/animal, shelters resembling their natural habitat, dirt floors for digging, and other elements of environmental enrichment (Manes, 2016; Van Zanten and Simpson, 2021). Although the animals are fed a diet specifically designed for this species (Vega Parry and Manes, 2000), maintaining a sustained mono diet for approximately 20 years, it is likely that they require supplementation of vitamins A, D, and E. Vitamin A deficiencies have been linked to stomatitis and inflammation of the upper respiratory tract (Herrera Ramírez, 2008; Rodríguez Molano, 2015). In this study, oral administration of vitamin A was chosen to coincide with the oral inspection of each patient, avoiding additional intramuscular injections that could be painful and stressful for the animal. Stress from increased frequency or duration of handling, including health management, predisposes the animals to illness and complicates the healing process (Doneley et al., 2018).

The immune response of reptiles is subjected to seasonal temperature-dependent alterations, with a maximum response observed when they are maintained near their preferred optimal body temperature (Mader, 2006; Doneley et al., 2018). Prolonged maintenance of reptiles at temperatures ranging from 15 °C to 21 °C predisposes them to opportunistic diseases. The innate and adaptive arms of the reptilian immune system are accepted to function optimally at the preferred body temperature of the animal. Many reptiles are kept at suboptimal temperatures, and simply warming them up can lead to significant improvements in their immunological defenses (Doneley et al., 2018). The monthly average temperature recorded in the region, where the breeding facility is located during the brumation period (May to September), was of 14.8 °C between 2018 and 2022 (EEAOC, 2023). This temperature would be at the limit of the suggested optimum for normal brumation (3.8 °C to 15 °C). Our results suggest that untimely winters with inappropriate temperatures for proper brumation may be a predisposing factor for stomatitis in captive *S. merianae*.

There is little knowledge about the immune system of reptiles. Some agents of reptiles are more or less infectious, but for most of them, the level of infectivity is unknown (Doneley et al., 2018). In our study, it is likely that *P. aeruginosa* acted as an opportunistic agent, and the great diversity of clinical

manifestations observed in our population may be attributed to the intrinsic immunity of each animal. The physiological state of each individual can impact susceptibility to diseases, even under similar captive conditions. Gravid females, males during breeding season, dominance of food sources, or other resources by enclosure mates, together with the effects of hierarchical stress, may predispose to illness (Meredith and Redrobe, 2012; Doneley et al., 2018).

CONCLUSIONS

This article provides a detailed report of clinical manifestations and macroscopic and histopathological findings in a captive population of *S. merianae* lizards with stomatitis. Identification of each individual facilitated precise treatment monitoring and the evolution of each patient. Furthermore, periodic follow-ups within the population have allowed us to suggest sanitary management measures to reduce the prevalence of this disease in captive animals.

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Author's Contributions

OLSL: Animal management, diagnosis and treatment of patients, drafting the manuscript. FHCC: Histopathological analysis, drafting the manuscript.

Competing interests

The author(s) declare that they have no competing interests.

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ECTOPIC PRIMARY ABDOMINAL PREGNANCY IN A PERSIAN CAT

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Abstract

Ectopic pregnancies are common in human medicine, while they are rarely recorded in animals. This report describes a case of 2.5-year-old ectopic primary abdominal pregnancy in a 7-year-old Persian cat. The cat was admitted to the surgery clinic for routine ovariohysterectomy with no characteristic clinical symptoms. During surgery, ovaries, uterus and ligaments were normal, like in a non-pregnant cat. Four intra-abdominal fetuses of different developmental stages were found and carefully removed. Three fetuses were found free in the abdomen inside their gestational sacs. The fourth fetus was covered with omentum and had no gestational sac. The gestational sacs had different sizes (2-8 cm in diameter). Two of the fetuses were fully-haired with normal position of limbs. No complications were recorded during the surgery and 10 months post-operative. This case report adds to the database of ectopic pregnancies in cats and further illustrates their incidental nature.

Key words: Abdominal pregnancy, cat, ectopic pregnancy, fetuses, ovariohysterectomy.

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VANMATERIČNI PRIMARNI ABDOMINALNI GRAVIDITET KOD PERSIJSKE MAČKE

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Kratak sadržaj

Vanmaterične trudnoće su česte u humanoj medicini, dok se retko beleže kod životinja. Ovaj izveštaj opisuje slučaj 2,5-godišnjeg vanmateričnog primarnog abdominalnog graviditeta kod 7-godišnje persijske mačke. Mačka je primljena na Kliniku za hirurgiju radi rutinske ovariohisterektomije bez karakterističnih kliničkih simptoma. Tokom operacije jajnici, maternica i ligamenti bili su normalni, kao kod mačke koja nije gravidna. Pronađena su i pažljivo izvađena četiri intraabdominalna fetusa različitih razvojnih stadijuma. Tri fetusa pronađena su slobodna u abdomenu unutar svojih gestacijskih vrećica. Četvrti fetus bio je prekriven omentumom i nije imao gestacijsku vreću. Gestacijske vreće bile su različite veličine (2-8 cm u promeru). Dva fetusa su bila sa potpuno formiranim dlačnim pokrivačem s normalnim položajem udova. Tokom operacije i 10 meseci nakon operacije nisu zabeležene komplikacije. Ovaj prikaz slučaja prestavlja dodatni podatak za bazu podataka o vanmateričnom graviditetu kod mačaka i dodatno ilustruje njihovu slučajnu prirodu.

Ključne reči: Abdominalni graviditet, mačka, vanmaterični graviditet, fetusi, ovariohisterektomija

INTRODUCTION

Ectopic pregnancy refers to a pregnancy occurring outside of the cavity of the uterus. While this disorder is common in humans, it is rarely recorded in animals (Vidiastuti et al., 2022). Ectopic pregnancies are classified according to the site of implantation and they can be abdominal and tubal pregnancies. The etiology and pathogenesis of ectopic pregnancy are not always clearly defined (Chong, 2017; Zheng et al., 2018; Jiasan et al., 2019).

Abdominal pregnancies are also subdivided into primary and secondary

forms. The primary form occurs when fertilized oocytes are released into the abdomen and implanted on the abdomen (Osenko and Tarello, 2014; Zheng et al., 2018; Vidiastuti et al., 2022). Secondary form occurs when a pregnant uterus is ruptured due to trauma or wounds and the fetuses are released into the abdominal cavity where they continue their development (Tirgari, 1986; Findik et al., 1998; Ivanova et al., 2019). Differentiation between primary and secondary extra-uterine pregnancies is controversial due to the presence of an intact (Dzięcioł et al., 2012), altered (Kumru et al., 2007) or partially missing reproductive system (Johnston et al., 1983). Consequently, the absence of signs of uterine rupture is one of the most important inclusion criteria for the diagnosis of primary extra-uterine pregnancies in cats (Osenko and Tarello, 2014).

Diagnosis of ectopic pregnancy is based mainly using X-ray (Johnston et al., 1983; Osenko and Tarello, 2014; Mirsepehr et al., 2015), ultrasound (Findik et al., 1998; Mirsepehr et al., 2015; Vidiastuti et al., 2022) and exploratory laparotomy (Bodle, 1979; Tirgari, 1986). Nevertheless, several cases of ectopic pregnancies were recorded in cats as an accidental finding during routine clinical examination or radiography (Mirsepehr et al., 2015; Chong, 2017; Ivanova et al., 2019). Moreover, the histological examination of the ectopic fetuses did not definitively prove their extra-uterine development (Rosset et al., 2011).

Death of the abdominal fetuses is a typical outcome of recorded ectopic pregnancies in cats due to insufficient nutrition resulting from a lack of adequate blood supply inside the abdomen (Mirsepehr et al., 2015; Chong, 2017; Zheng et al., 2018). Therefore, laparotomy, removal of the ectopic fetuses and ovariohysterectomy are usually performed for treatment and prevention of recurrence of ectopic pregnancies in cats (Mirsepehr et al., 2015; Chong, 2017; Zheng et al., 2018; Ivanova et al., 2019). Nevertheless, spontaneous resolution of early ectopic pregnancies may occur, suggesting that many diagnosed cases in early stages require no treatment at all (Mirsepehr et al., 2015; Chong, 2017).

Ectopic pregnancy in cats has been recorded in the veterinary literature. However, it is quite uncommon, as shown in Table 1. Therefore, this case report adds to the available database of ectopic pregnancies in cats and further explains their accidental nature.

Table 1. Types of ectopic pregnancies recorded in different breeds of cats and characters of the ectopic fetuses

Age/Breed	Type of ectopic pregnancy	Characters of the fetuses	Reference
A 3-year-old Persian cat	Primary abdominal pregnancy	One mummified fetus, 7.25 x 4.74 cm	Vidiastuti et al. (2022)
Two European shorthair cats	Secondary abdominal pregnancies	Three fetuses, 3 cm in diameter Two, 6 × 3.5 cm and 5 × 4.5 cm	Ivanova et al. (2019)
A British shorthair	Tubal pregnancy	One fetus, not identified	Jiasan et al. (2019)
Unidentified cat A British shorthair cat	Primary abdominal pregnancy Tubal pregnancy	One calcified fetus, 4 cm in diameter One immature fetus, unidentified size	Zheng et al. (2018)
A domestic, short-haired cat	Secondary abdominal pregnancy	Two fetuses, 10 cm from crown to rump	Chong (2017)
A domestic short-haired cat	Secondary abdominal pregnancy	Three fetuses, fully developed	Mirsepehr et al. (2015)
A domestic short haired spayed cat	Primary abdominal pregnancy	Three calcified fetuses, 4-5 cm in size	Osenko and Tarello (2014)
A domestic shorthair Unidentified cat	Secondary abdominal pregnancies	Two fetuses, different stages of development One outside the uterus and one inside, 30-35 days pregnancy	Dzięcioł et al. (2012)
A 1.5-year-old domestic short-haired cat	Secondary abdominal pregnancies	One mummified fetus, 7 cm in length	Rosset et al. (2011)
A 2-year-old crossbreed free-roaming cat	Secondary abdominal pregnancies	One dead fetus at 55 days' gestation	Kumru et al. (2007)
Angora cat	Secondary abdominal pregnancy	One fetus, 6 x 2.5 cm	Findik et al. (1998)
A 2.5- year-old short-haired cat	Secondary abdominal pregnancy	One encapsulated fetus, 7 x 4 x 4 cm and one embedded in the omentum, 7.5 x 3 cm	Tirgari (1986)

Age/Breed	Type of ectopic pregnancy	Characters of the fetuses	Reference
A 2.5- year-old domestic short-haired cat	Secondary abdominal pregnancy	Two necrotic fetuses, 7 x 4 cm	Johnston et al. (1983)
Unidentified cat	Primary abdominal pregnancy	Three mummified fetuses, 2,3 and 6 cm in diameter	Bodle (1979)

CASE PRESENTATION

This study was approved by the ethical committee at Faculty of Veterinary Medicine, Cairo University, Egypt. The cat was treated in accordance with guidelines established by the international and institutional Animal Care and Use Committees.

A seven-year-old Persian cat was brought to the veterinary clinic for routine ovariohysterectomy. The owner adopted the cat when it was 2.5 years old from the street. The cat showed normal sings of regular estrous cycles. Detailed history of previous pregnancies could not be obtained. During the preoperative examination, two large intra-abdominal hard masses were palpated in the middle abdomen and no other abnormalities were detected. The masses were freely mobile inside the abdominal cavity.

The cat was given general anesthetic for ovariohysterectomy and exploratory laparotomy. During surgery, three freely movable encapsulated intra-abdominal masses were found and easily removed. A fourth mass covered with omentum was also removed by careful blunt dissection. The ovaries, uterus and ligaments had normal appearance without any abnormalities. Ovariohysterectomy was carried out according to a standard procedure. Prior to closing, the abdominal cavity was thoroughly examined and washed with sterile warm normal saline solution. The cat was monitored for 10 months after surgery. A successful recovery without any complications was reported.

The dimensions of the removed masses were 8 x 5 x 4 cm, 6 x 5 x 3cm, 4 x 2 x 2 cm and 1.5 x 1 x 0.5 cm as shown in Figure 1. Three of these masses were covered with calcified sacs while the fourth mass was covered with omentum and had no sac. After opening the sacs of the three masses and removal of the omental tissue from the fourth mass, four mummified and calcified fetuses were found. Two fetuses were well-developed, fully haired and had normal positions of limbs (Figure 2). One of the well-developed fetuses had a normal body, while the second one had open abdomen without viscera as shown in Figure 2.

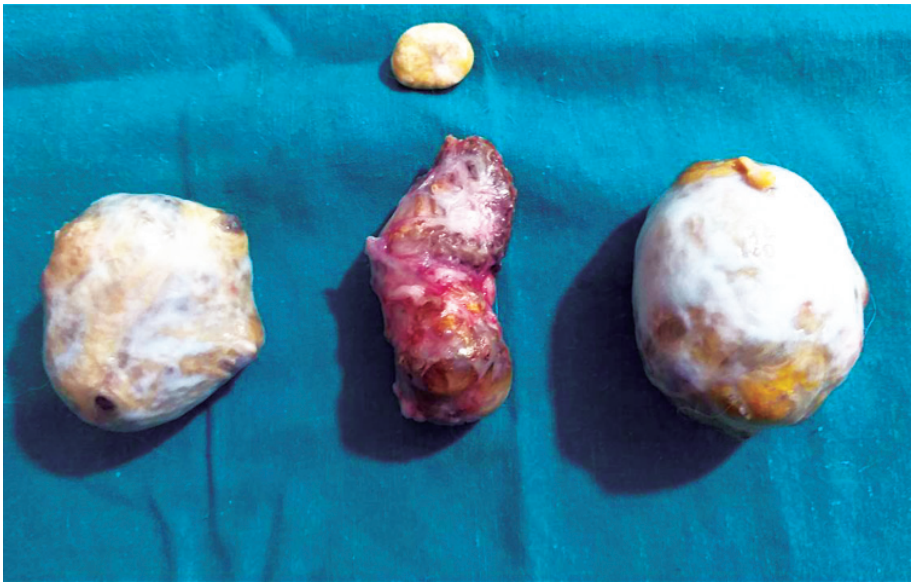

Figure 1. Four masses of different sizes were removed from the abdominal cavity of the cat. Note that three of them were encapsulated by gestational sacs and one embedded in the omentum.

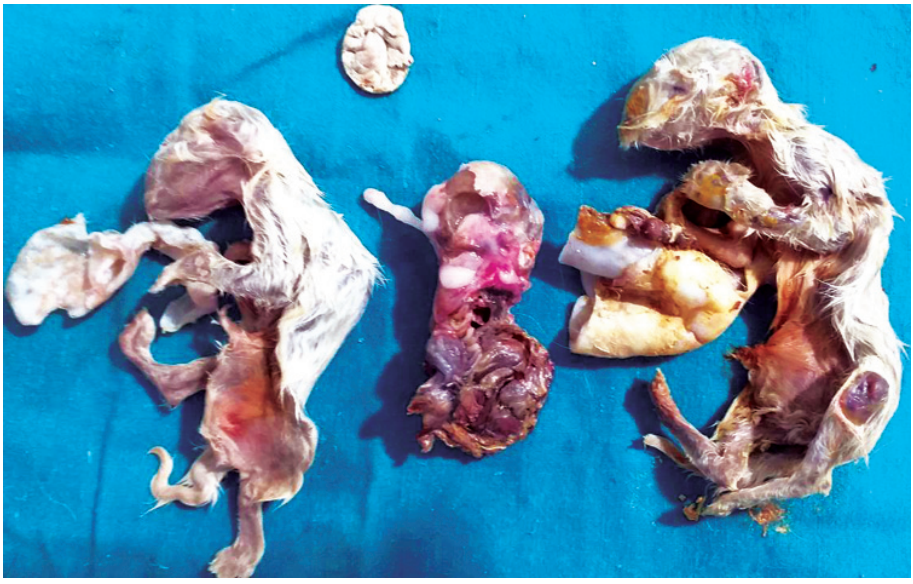

Figure 2. Four ectopic fetuses at different stages of development were identified after opening the gestational sacs and removal of the omentum.

DISCUSSION

While ectopic pregnancy is a common pathology in humans, it is still rarely recorded in veterinary literature. No detailed epidemiological studies on ectopic pregnancy have been conducted on animals, particularly cats. Cats are less likely to have ectopic pregnancy due to a difference in their endotheliochorial placentation (Zheng et al., 2018). This report describes a rare case of long-standing (> 2.5 years) ectopic primary abdominal pregnancy in a Persian cat.

Diagnosis of this case depended on the case history and findings of the clinical examination as well as laparotomy. In addition, abdominal ectopic pregnancy is truly primary when placentation presents onto a peritoneal or omental surface (Zheng et al., 2018; Vidiastuti et al., 2022), as in the current case. The differences between this case and other recorded ectopic pregnancy cases in cats are the number (N = 4) and different developmental stages of the ectopic fetuses as shown in Table 1.

Regarding the cat breed, domestic short-haired cats were the most commonly affected breed with ectopic pregnancies as shown in Table 1. However, the reported cat in this case study is a Persian cat. Similarly, a Persian cat with ectopic pregnancy was recently diagnosed by Vidiastuti et al. (2022).

Similar to the current cat, cats with ectopic pregnancy generally exhibit no clear clinical signs due to the aseptic condition of the ectopic fetuses which can remain within the animal's body for months or even years without complications (> 2.5 years). This is in agreement with the previously reported results by other authors (Dzięcioł et al., 2012; Zheng et al., 2018). Nevertheless, some cats with ectopic pregnancies, particularly secondary abdominal ectopic pregnancies, show various clinical signs such as loss of appetite, fever, vomiting, vaginal bleeding and peritonitis (Rosset et al., 2011; Dzięcioł et al., 2012; Mirsepehr et al., 2015). In other cases, particularly in primary abdominal ectopic pregnancies, cats have no clinical signs and they are detected during abdominal radiography or routine spaying as in the current case. Similar findings have been recorded before (Osenko and Tarello, 2014; Ivanova et al., 2019; Vidiastuti et al., 2022). Therefore, ectopic pregnancy may be detected as an incidental finding during clinical examination prior to ovariohysterectomy, like in the present case. The lack of associated clinical signs appears to demonstrate that such pregnancy is compatible with a normal healthy life in cats. Similar finding was confirmed by other authors (Chong, 2017; Zheng et al., 2018; Ivanova et al., 2019).

In the present case, only two of the four fetuses were palpable prior to the surgery. This could be attributed to the small size of the other two fetuses. This

finding is similar to the findings of other recorded cases of ectopic pregnancies in cats (Dzięcioł et al., 2012; Osenko and Tarello, 2014; Zheng et al., 2018; Ivanova et al., 2019). Therefore, radiography and ultrasonography examinations are useful tools for diagnosis of this problem in cats (Osenko and Tarello, 2014; Zheng et al., 2018). The main limitation in the present case report is the lack of radiography and ultrasonography examinations due to their unavailability at the time of examination.

During surgery, the uterus, ovaries and ligaments were normal in the present case. Therefore, it was diagnosed as primary abdominal pregnancy. Similar findings were recorded by other authors (Zheng et al., 2018; Ivanova et al., 2019). Unlike in these findings, ectopic abdominal fetuses secondary to trauma were observed in cats with multiple uterine abnormalities (Johnston et al., 1983; Dzięcioł et al., 2012; Chong, 2017). Moreover, Zheng et al. (2018) and Jiasan et al. (2019) diagnosed tubal ectopic pregnancy in the fallopian tubes of cats, and Osenko and Tarello (2014) recorded a case of ectopic pregnancy in a spayed cat at necropsy.

In the current case, laparotomy, removal of the ectopic fetuses and ovariohysterectomy were performed for treatment and prevention of recurrence of ectopic pregnancy. The surgeries were simple and without any early or late complications. This can be explained by the lack of attachment of the fetuses to any of the internal organs. These findings are in accordance with the findings of other authors (Mirsepehr et al., 2015; Chong, 2017; Zheng et al., 2018; Ivanova et al., 2019).

The number of extra-uterine fetuses ranged between one and three as shown in Table 1. The cat had four extra-uterine fetuses. According to the available literature, this is the largest number of extra-uterine fetuses recorded in cats to this day. In addition, the removed fetuses here were grown to different stages of development. The difference in developmental stages of the removed fetuses can be explained by the difference in the time of death of each fetus. Therefore, there was a variation in the size of the removed ectopic fetuses. Development of abdominal fetuses to advanced stages without an elaborated placentation has been recorded previously in cats (Mirsepehr et al., 2015).

According to Knospe (2002), the removed ectopic fetuses died between three and eight weeks of gestation. At 60 days of gestation, pigmentation can occur on the skin, hair, and nails, the brain and bones ossification also are present at this time. Signs of fetal mummification and calcification were observed in all ectopic fetuses. This is the common end of all recorded cases of ectopic pregnancies in cats due to malnutrition of the ectopic fetuses (Mirsepehr et al., 2015).

CONCLUSION

This is an interesting rare case of long-lasting ectopic primary abdominal pregnancy in a Persian cat. Four extra-uterine fetuses at different stages of development were present inside the abdomen for more than 2.5 years without any clinical signs or complications.

Competing interest

The author declares no conflicts of interest affecting the work reported in this paper. This research received no external funding.

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COMBATTING METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) IN THE FOOD INDUSTRY BY HARNESSING THE POWER OF NATURE: A SYSTEMATIC REVIEW

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Abstract

Antibiotic resistance is a critical global health concern, with *Methicillin-resistant Staphylococcus aureus (MRSA)* posing a significant challenge due to its resistance to commonly used antibiotics. Recent research has revealed the potential of natural compounds and microorganisms in combatting *MRSA* and other antibiotic-resistant bacteria. In this systematic review, we studied the effect of essential oils, bacteriophages, bacteriocins, and probiotics on *S. aureus,* including *MRSA* in particular, in the food industry. Essential oils (EOs) have gained significant attention because of their antimicrobial properties, inhibiting *MRSA* growth by damaging bacterial cells and inhibiting essential enzymes and compounds. Cinnamon oil liposomes caused the most significant decrease in *MRSA* populations among our reviewed essential oils. Bacteriophages can lyse the bacterial host. They encode peptidoglycan hydrolases called endolysins that target the bacterial cell wall. In our study, *S. aureus* phage (containing *CHAPLysGH15* and *LysGH15*), and phage *SA11* endolysin *(LysSA11*) were the most effective against *S. aureus*. Bacteriocins, antimicrobial peptides produced by bacteria, also show potential in combatting *MRSA*, mainly by generating organic acids that interfere with bacterial metabolism. According to our review, the

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most effective bacteriocins against *S. aureus* were *Enterocin AS-48* with phenolic compounds or with *2NPOH*, Bacteriocin isolated from *Lactobacillus pentosus - Pentocin JL-1*, and bacteriocin produced by *S. pasteuri RSP-1,* respectively. Probiotics can compete with pathogens by producing antimicrobial compounds that disrupt *MRSA* cell production and ultimately lead to bacterial death. In our review, the most effective probiotics were *Streptomyces griseus*, *Pediococcus acidilactici strains A11 and C12*, *Lactococcus lactis*, and *Lactobionic acid* respectively. A multi-hurdle approach combining these natural agents has shown promising results in targeting and eliminating *MRSA* cells. By harnessing the power of nature, we can potentially overcome the challenges posed by *MRSA* and other antibiotic-resistant bacteria.

Key words: *Methicillin-resistant Staphylococcus aureus (MRSA)*, Essential oils, Bacteriophage, Bacteriocin, Probiotic

BORBA PROTIV STAPHILOCOCCUS AUREUS-a (MRSA) OTPORNOG NA METICILIN U PREHRAMBENOJ INDUSTRIJI KORIŠĆENJEM SNAGE PRIRODE: SISTEMATSKI PREGLEDNI RAD

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Kratak sadržaj

Otpornost na antibiotike je globalni zdravstveni problem, a meticilin rezistentan *Staphilococcus aureus* (MRSA) predstavlja značajan izazov zbog otpornosti na antibiotike koji se obično koriste. Skorija istraživanja otkrila su potencijal prirodnih jedinjenja i mikroorganizama u borbi protiv MRSA i drugih bakterija otpornih na antibiotike. U ovom preglednom radu proučavan je efekat eteričnih ulja, bakteriofaga, bakteriocina i probiotika na *S. aureus*, uključujući i izolate MRSA, u prehrambenoj industriji. Eterična ulja (EO) privukla su značajnu pažnju zbog svojih antimikrobnih svojstava, inhibirajući rast MRSA tako što oštećuju bakterijsku ćeliju i inhibiraju njihove esencijalne enzime i jedinjenja. Od svih ispitanih eteričnih ulja, lipo-

zomi ulja cimeta doveli su do najznačajnijeg smanjenja populacije MRSA. Bakteriofagi mogu da liziraju bakteriju koju napadaju. Oni sintetišu enzime peptiodoglikan hidrolaze koji su poznati pod nazivom - endolizini, koji oštećuju bakterijski zid. U našoj studiji, *S. aureus* fage (koje sadrže CHAPLisGH15 i LisGH15) i fag SA11 endolizin (LisSA11) bili su najefikasniji protiv *S. aureus*. Bakteriocini, antimikrobni peptidi koje proizvode bakterije, takođe pokazuju potencijal u borbi protiv MRSA, uglavnom stvaranjem organskih kiselina koje ometaju metabolizam bakterija. Na osnovu rezultata našeg preglednog rada, najefikasniji bakteriocini protiv *S. aureus* su bili Enterocin AS-48 sa fenolnim jedinjenjima ili sa 2NPOH, Bacteriocin izolovan iz *Lactobacillus pentosus* - Pentocin JL-1 i bakteriocin proizveden od *S. pasteuri* RSP-1. Probiotici mogu da deluju na patogen tako što proizvode antimikrobna jedinjenja koja ometaju proizvodnju MRSA ćelija i na kraju dovode do smrti bakterija. U našem pregledom radu, najveću efikasnost pokazali su probiotici *Streptomices griseus*, *Pediococcus acidilacti* sojevi A11 i C12, *Lactococcus lactis* i *Lactobionic acid*. Pristup koji kombinuje ove prirodne agense pokazao je zadovoljavajuće rezultate u prepoznavanju i eliminaciji MRSA ćelija. Koristeći snagu prirode, razvijamo potencijal za prevazilaženje infekcija uzrokovanih sa MRSA-ma i drugim bakterijama koje su otporne na antibiotike.

Ključne reči: *Staphilococcus aureus* otporan na meticilin (MRSA), eterična ulja, bakteriofag, bakteriocin, probiotik

INTRODUCTION

Staphylococcus aureus is a gram-positive pathogenic bacterium. The ability of *S. aureus* to adhere to specific host substrates and evade host defenses (Eom, et al. 2014; Lu, et al. 2021), as well as its ability to survive in various environmental conditions while posing different virulence factors (de Oliveira, et al. 2010; Eom, et al. 2014; Burris, et al. 2015; Lu, et al. 2021), makes it highly virulent and capable of causing life-threatening infections in both humans and animals (Zhu, et al. 2015; Catteau, et al. 2017; Lalouckova, et al. 2021). Foodborne diseases caused by *S. aureus* (Lee, et al. 2009; de Oliveira, et al. 2010; Keyvan and Tutun 2019; Prastiyanto, et al. 2020; Lalouckova, et al. 2021) are generally limited to food poisoning and gastroenteritis, resulting from enterotoxins produced by *S. aureus* (Lee, et al. 2009; Zhu, et al. 2015; AL-Saadi 2016; Prastiyanto, et al. 2020).

Antibiotic resistance is one of the most significant health challenges of the

century (Lee, et al. 2009; Chang, et al. 2017; Chang, et al. 2017; Prastiyanto, et al. 2020). Antibiotic-resistant forms of *S. aureus*, such as methicillin-resistant *Staphylococcus aureus (MRSA)*, are multi-drug-resistant (Eom, et al. 2014; AL-Saadi 2016; Lu, et al. 2021) to β-lactam antibiotics (Eom, et al. 2014; Redwan et al. 2016; Catteau, et al. 2017; Lestari, et al. 2019; Prastiyanto, et al. 2020). Foodborne *MRSA* is a major concern for public health worldwide (Lee, et al. 2013; Redwan, et al. 2016; Kang, et al. 2020; Lu, et al. 2021), because it can enter the food chain as animal based food (Vaiyapuri, et al. 2019; Kang, et al. 2020) or by colonizing in food handlers and transferring from them to food (Eom, et al. 2014). A high rate of morbidity and mortality by *MRSA* have been reported worldwide (Zhu, et al. 2015; Redwan, et al. 2016; Zouhir, et al. 2016; Salem 2017; Zihadi, et al. 2019). *MRSA* has already been isolated from food, indicating that it is present as a contaminant in the food production chain (Ansari, et al. 2020; Afshari, et al. 2022). The presence of *MRSA* has been reported mainly in meat such as pork, beef, lamb, chicken, rabbit, and turkey, as well as in dairy products such as milk and cheese (Mohammed-Ali, et al 2015). This means that the food production chain is a pathway of transmission between resistant microorganisms and humans (Mohammed-Ali, et al 2015). Food safety is an important global concern in the food industry and public health. Many preservatives that are used to control microbial growth in foods not only increase the shelf-life of food products, but they also reduce the incidence of foodborne diseases (Xu, et al. 2016; Chang, et al. 2017). Due to consumer worries regarding safety of chemical preservatives utilized in food, there is an increasing need for natural alternatives that can function as food preservatives. (Gyawali, et al 2014). Therefore, it is critical to use natural agents that control or prevent foodborne pathogens, including *MRSA*, in food (Kang, et al. 2020). By utilizing these natural antimicrobials as food preservatives, the need for excessive physical and chemical food processing can be reduced while ensuring microbial safety and environmental preservation (Yusuf, 2018).

Several natural compounds from plants, animals, and microorganisms have been studied and applied in order to inhibit or control the growth of foodborne microorganisms, including *MRSA*. Plant-derived essential oils are commonly used as flavoring and preservation agents in food and drinks have antimicrobial and antioxidative activity (Cui, et al. 2018; Yuan, et al 2018).

Bacteriophages are viruses that infect bacteria and can exhibit inhibitory activity against *S. aureus*, particularly *MRSA*. Furthermore, since gram-positive bacteria lack an outer membrane, bacteriophages can directly lyse the cell wall from the outside (Lysis from without) (Lu, et al. 2021). Bacteriocins are proteins that exhibit bactericidal effects on a variety of bacteria, including *S. aureus*. They are considered as alternatives to traditional antibiotics (Zhu, et al.

2015; Chauhan, et al. 2017; Lestari, et al. 2019) and an effective approach for use in food against *MRSA* (Arumugam, et al. 2019). Probiotics are living organisms used as food additives to help maintain a healthy microbial balance in the gastrointestinal tract, leading to better health in humans (Lee, et al. 2021).

This review focuses on the effective natural antimicrobials originating from plants and microorganisms against *MRSA*, including essential oils, bacteriophages, bacteriocins, and probiotics. The mechanisms of action, as well as their effectiveness, are also surveyed. Our main aim was to review the efficiency of natural antimicrobial agents in combating *MRSA* in food.

MATERIAL AND METHODS

Study Design

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines were applied to conduct this systematic review. The main objective of this study was to review the literature on natural approaches for controlling *MRSA* in food.

Search Strategy

In this study, the main databases, including Scopus, PubMed, Google Scholar, and Science Direct, were searched. The literature review was limited to studies published from 2000 to 2023. The search was independently conducted for each database, focusing on controlling *Methicillin-resistant Staphylococcus aureus* OR *MRSA* in any food product worldwide. The keywords used were "*Methicillin-resistant Staphylococcus aureus*" OR "*MRSA*" AND "Dairy" OR "Milk" OR "Meat" OR "Food" AND "Essential Oils" OR "Probiotic" OR "Bacteriophage" OR "Bacteriocin" AND "Control".

Inclusion and Exclusion Criteria

This review included articles (n = 83) that reported on the natural types of effective antimicrobials, including essential oils, bacteriophages, bacteriocins, and probiotics, against *MRSA*. The selection for inclusion eligibility was conducted by scanning the titles, abstracts, and full texts of retrieved articles. The focus of our study was on livestock-associated *(LA) MRSA*. All review studies, duplicate publications, as well as clinical reports and trials on healthcare-associated *(HA) MRSA*, were excluded.

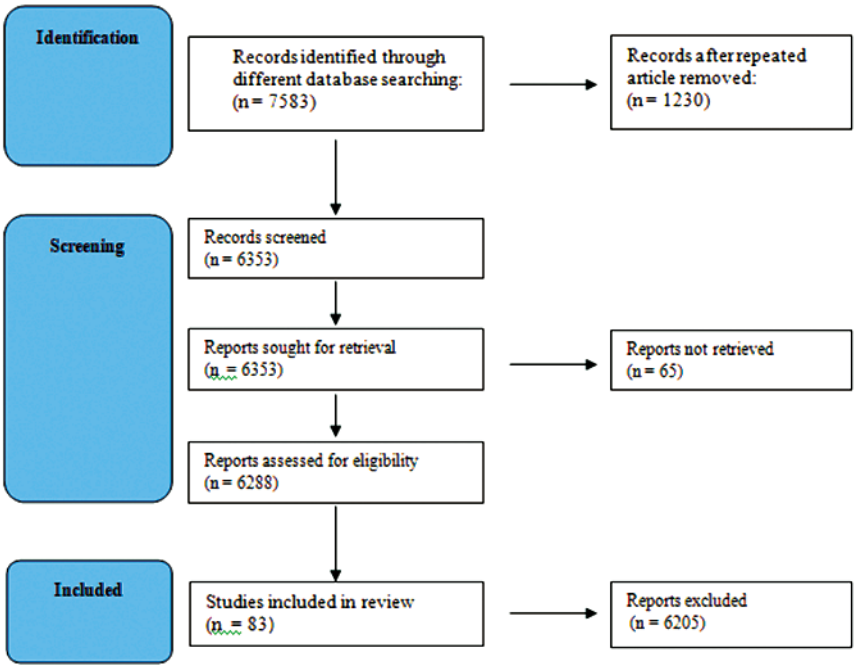

Figure 1. PRISMA flowchart for studies selection

RESULTS

Essential Oils

Essential oils have shown an antimicrobial effect against *S. aureus* and *MRSA* in particular. For instance, Cinnamon oil, Thyme oil, and Lemongrass oil reduced the *MRSA* population in minced meat by 7.6, 6.53, and 5.94 log CFU/g, respectively, when applied at a concentration of 1.5% (Eom, et al. 2014). Cinnamon oil was bactericidal against the biofilm activity of *MRSA*. A concentration of 1.0 mg/mL of cinnamon oil was sufficient to eliminate *MRSA* biofilm (Cui, et al. 2016). *Syzygium aromaticum (CLV)* and *Cinnamomum zeylanicum (CIN)* exhibited bactericidal activity at a concentration of 200 µg/mL against *S. aureus* and reduced the population of *S. aureus* by 4.50 log10 CFU/mL and 3.97 log10 CFU/mL, respectively (Mandal, et al. 2011).*Cuminum cyminum (CMN)* exhibited bactericidal activity at 300 µg/mL and caused a reduction of 0.59 log10 CFU/mL in *MRSA* after 24 hrs (Mandal, et al. 2011). The MIC concentration of the polyphenolic components of green tea, neem leaves extract, and a combination of green tea and neem were 15.62, 31.25,

and 46.87 mg/mL, respectively (Zihadi, et al. 2019). Allicin liquid was active against *S. aureus* strains, and all *MRSA* strains were inhibited by *allicin* at 32 µg/mL (Cutler, et al 2004). The indigenous cinnamon B leaf oil *(Cinnamomum osmophloeum)* had antibacterial effect against *MRSA* with an MIC of 250 µg/mL (Chang, et al. 2001). The *ethanolic* extract of *Elettaria cardamomum* displayed antibacterial activity against *MRSA*, with a minimum inhibitory concentration (MIC) of 0.25 mg/disk and minimum bactericidal concentration (MBC) of 0.50 mg/disk against *S. aureus* (Yassin, et al. 2022). *Nigella sativa* oil extract was effective against *MRSA*, with inhibition zones of 7 ± 1 mm and 10 ± 0.9 mm observed at concentrations of 400 µl and 800 µl, respectively (Abdullah, et al. 2021). *Litsea cubeba* essential oil *(LC-EO)* contained high percentages of *aldehydes*, *primarily β-Citral* (39.25%) and *α-Citral* (30.89%). *LC-EO* caused a steady decrease in *MRSA* populations, with a 99.99% reduction observed after 2 hours of treatment with 0.25 mg/mL of *LC-EO* (Hu, et al. 2019). *Red propolis* extracts *(RPE)* had an inhibition zone of 16.5 ± 0.5 mm and 19.3 ± 0.5 mm against *S. aureus* and *MRSA*, respectively (Zhang, et al. 2022). The essential oil extracted from *Carum carvi L.* seeds completely prevented *MRSA* biofilm formation at a concentration of 1.28%, with decreasing inhibitory effects observed at lower concentrations (Liu, et al. 2023). The *ethanol* extract from *Psoralea corylifolia* seeds exhibited antibacterial activity against Gram-positive bacteria, with inhibition zones of 14 mm and 16 mm for *S. aureus* and *MRSA,* respectively. *MRSA* cells treated with 1600 µg/mL of the extract were deformed and collapsed (Li, et al. 2019). *Backhousia citriodora* essential oil significantly inhibited 90.01% to 93.39% of *S. aureus* biofilms (Lim, et al. 2022). Oregano *(Origanum vulgare)* and clove *(Eugenia caryophyllata)* essential oils were effective against *S. aureus* and *MRSA*, with complete inhibition at concentrations of 0.63 µg/mL and 10 µg/mL, respectively (Debiagi, et al. 2020). Finally, *Lippia micromera* and *Plectranthus amboinicus* exhibited potent antibacterial activity against *MRSA*, with large inhibition zones of 23.7 - 35.7 mm (Bugayong, et al. 2019). The MIC of a combination of *oregano* and *thyme* essential oils was found to be 320 µg/mL (Boskovic et al. 2015) .Other studies reporting the effectiveness of essential oils are summarized in Table 1. According to Table 1, the most effective compound against *MRSA* is the *liposome* containing cinnamon oil, with a MIC of 0.25 mg/mL and MBC of 0.25 mg/mL, this compound resulted in a 99.99% decrease in *MRSA* populations after 4 hours and a decrease of 2.83 logs after 24 hours at a concentration of 1.0 mg/mL. Other most effective EOs against *MRSA* are *Cinnamomum zeylanicum*, *Syzygium aromaticum*, *Cuminum cyminum*, respectively. Additionally, *allicin*, *glabrol*, *clove buds*, and *Backhousia citriodora* essential oil s < 0, have shown significant effectiveness against *MRSA* with low MIC values.

Table 1. Effectiveness of essential oils against *MRSA*

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Cinnamon oil, Thyme oil and Lemongrass oil	*Cinnamaldehyde, eugenol, Alpha* and *beta-citral, mycrene*	The initial count of *MRSA* after inoculation (at zero time) was 10.28(log CFU/g) which at a concentration of 1.5% Cinnamon oil, Thyme oil and Lemongrass oil reduced *MRSA* population by 7.6, 6.53, 5.94 log CFU/g, respectively.	(Salem 2017)
Liposome containing cinnamon oil	*Cinnamon oil*	After 4h there was a decrease of around 99.99% in the *MRSA* populations and after 24 h, the population of *MRSA* decreased by 2.83 logs using 1.0 mg mL^{-1}. MIC (mg/mL): 0.25, MBC (mg/mL): 0.25	(Cui, et al. 2016)
Indian Spices	*Syzygium aromaticum (CLV), Cinnamomum zeylanicum (CIN) and Cuminum cyminum (CMN)*	After 24 h the *CIN* and *CLV* showed bactericidal activity at concentration 200 μg/mL against *S. aureus* reducing 4.50 log10 cfu/mL and 3.97 log10 cfu/mL, respectively; *CMN* exhibited bactericidal effects at 300 μg/mL, leaving 0.59 log10 cfu/mL. Effectiveness order: *C.zeylanicum* > *S.aromaticum* > *C. cyminum*	(Mandal, et al. 2011)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Polyphenolic components of *Green tea,* Neem leaves extract of *Camellia sinensis* and *Azadirachta indica leaves*	*Catechin*	MIC (mg/mL): *Green tea*: 15.62 *Neem*: 31.25 *Green tea + Neem*: 46.87 (*green tea* extract is more potent than *neem* against *MRSA*)	(Zihadi, et al. 2019)
Allicin	NR	MIC: 32 µg/Ml, MBC: 128 µg/mL	(Cutler, et al 2004)
Cinnamomum osmophloeum leaf	*Cinnamaldehyde*	MIC: 250 µg/mL	(Chang, et al. 2001)
Thyme (Tymus vulgaris) and Oregano (Origanum vulgare)	*Thymol* from *Thyme and Carvacrol* from *Oregano*	MIC of *Oregano* and *Thyme*: 320 µg/mL MBC of *Oregano*: 1280 µg/mL MBC of *Thyme*: 640 µg/mL	(Boskovic, et al. 2015)
PFF (phlorofucofuroecko, a marine-derived polyphenol found in brown algae)	NR	MIC: 64 µg/mL	(Eom, et al. 2014)
URS (ursolic acid 3-O-α-L arabinopyranoside was isolated from the leaves of A. henryi (Oliv) with oxacillin	Ursolic acid 3-O-α-L-arabinopyranoside with (URS) oxacillin	MIC: 6.25 µg/mL After 24 h, treatment with 1/2 MIC OXA and 3/4 MIC URS in combination resulted in combined group bacteria counts that decreased to 3 log10.	(Zhou, et al. 2017)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Pink oyster mushroom Pleurotus flabellatus	The terpenoid compound group	MIC: 62.5 mg/mL MBC: 250 mg/mL	(Ghosh, et al. 2016)
Carvacrol	NR	MIC: 0.11 mg/mL	(Keyvan, and Tutun 2019)
Bulb Eleutherine Americana	Naphtho-quinone	MIC: 125-500 g/mL, MBC: 250-1000 g/mL	(Ifesan, et al. 2009)
Aloysia citriodora essential oils from Baqa al-Gharbiyye and Umm al-Fahm	lipophilic structures like α- citral and α-curcumene	MIC: 2.5 µg/mL	(Arumugam, et al. 2019)
Garlic	Allicin (allyl 2-propenethiosulphinate)	MIC: 256 g/mL	(Prastiyanto, et al. 2020)
Cinnamon (Cinnamomum verum)	Cinnamaldehyde, eugenol	Against MSSA: MIC of 250 µg/mL Against MRSA: MIC of 250 µg/mL	(Prastiyanto, et al. 2020)
Thyme (Thymus vulgaris L.)	Thymol, carvacrol	MIC of 0.25% (v/v)	(Prastiyanto, et al. 2020)
Clove (Eugenia caryophyllata)	Eugenol, Cariofilene	MIC of 0.25% (v/v)	(Prastiyanto, et al. 2020)
Rosemary (Rosemarinus officinalis)	Borneol, 1, 8-cineole	MIC of 1.0% (v/v) MIC of 0.5% (v/v)	(Prastiyanto, et al. 2020)
Sage (Salvia officinalis)	Thujone, cineol, thymol	MIC of 1.0 (% v/v)	(Prastiyanto, A et al. 2020)
Tea tree (Melaleuca alternifolia)	Terpene	MIC of 0.5% (v/v)	(Prastiyanto, et al. 2020)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Flavonoids from licorice	glabrol, licochalcone A, licochalcone C, and licochalcone E	After 3 h, at 8 mg/mL killed both MRSA T144 and MSSA ATCC29213 completely and after 1 h, all MRSA T144 and MSSA ATCC29213 cells were killed after exposure to glabrol at 4–16 mg/mL.	(Burgos, et al. 2015)
Clove buds	Eugenol	MIC: 0.62 mg/mL	(Xu, et al. 2016)
Chuzhou chrysanthemum	B-Eudesmene, L-Borneol, Camphor	MIC: 5 mg/mL, MBC: 10 mg/mL	(Cui, et al. 2018)
Syzygium antisepticum plant	b-caryophyllene	MIC: 0.12 mg/mL MBC: 0.5 mg/mL	(Yuan, et al 2018)
Sanguisorba officinalis strains	Ethanol	At the concentration of 10 mg/mL S. officinalis the growth of the MRSA was inhibited. at a low concentration (<2.5 mg/mL), inhibitory effect of S. officinalis on biofilm formation in the MRSA strain was obvious.	(Chen, et al. 2015)
Korean soybean fermented product doenjang	Methanolic	MIC: 2048 μg/mL	(Lalouckova, et al. 2021)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Bisdemethoxycurcumin with three antibiotics (gentamicin, ampicillin and oxacillin)	NR	MIC: 7/8 µg /mL for all S. aureus strains including MRSA. The combination of BDMC with antibiotics caused more than 3 log10 cfu/mL reductions on all the three S. aureus strains.	(Hermawati, et al. 2016)
Thymol and carvacrol with organic acids (lactic acid)	NR	Combination of thymol and carvacrol with organic acids results a reduction over two log cycles in initial bacterial after 24 h.Thymol and carvacrol showed MIC of 0.6 and 1.25 µL/mL and MIC of lactic acid was 2.5 µL/mL	(de Oliveira, et al. 2010)
Elettaria cardamomum ethanolic extract	a-terpinyl acetate and 1,8 cineole	MIC: 0.25 mg/disk, MBC: 0.50 mg/disk	(Yassin, et al. 2022)
Nigella sativa (Black seed) Oil	Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL and Pentane, 2-cyclopropyl	MIC shows that at the concentration of 400 µl with (7± 1) mm of inhibition zone and 800mL concentration was (10± 0.9) mm	(Abdullah, et al. 2021)
Litsea cubeba essential oil	β-Citral and α-Citral	MIC 0.5 mg/ mL, MBC 1.0 mg/ mL	(Hu, et al. 2019)
Red Propolis	Pinobanksin, pinobanksin-3-acetate	MIC: of 50 µg/mL MBC: 200 µg/mL	(Zhang, et al. 2022)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Essential Oil Extracted from Carum carvi L. seeds (CEO)	Carvone and limonene	MIC: 6.4 µg/mL	(Liu, et al. 2023)
Ethanol Extracts of Psoralea corylifolia Seeds	Phenol, hydrazine, aldehyde, and ketone	MIC: 50 µg/mL MBC: 100 µg/mL	(Li, et al. 2019)
Black seed (Nigella sativa) oil	Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL and Pentane, 2-cyclopropyl	MIC: 32.8 mg/mL MBC 42.2 mg/mL	(Abdullah, et al. 2021)
Backhousia citriodora Essential Oil (BCEO) leaves	oxygenated monoterpenes and neral phytocompounds	MIC: 6.25 µL/mL, MBC: 50 µL/mL	(Lim, et al. 2022)
Pelargonium graveolens Oil	citronellol, citronellyl formate	MIC: 1.56 µg/mL.	(Jaradat, et al. 2022)
Origanum-vulgare and Eugenia caryophyllata oil	phenols components	MIC CEO: 10 µg/mL MIC OEO: 0.63 µg/mL	(Debiagi, et al. 2020)
Essential Oils from Leaves of Some Aromatic Plants	Monoterpenes	MIC :2.00 %, MBC>4.00 %	(Bugayong, et al. 2019)

Essential oils			
Name	**Active Components**	**Effectiveness**	**References**
Essential Oils from Elettaria Cardamomum fruit capsules	monoterpenes and sesquiterpenes	MIC: 250 μg/mL.	(Jha, et al. 2022)

NR: not reported

Bacteriophage

A phage endolysin, *LysP108*, was able to decrease viable *MRSA* cells by approximately 2 log units within 30 minutes at an optimal concentration of 250 μg/mL. At an MIC of 100 μg/mL, while the antibiofilm activity of the endolysin resulted in the removal of 66% of *MRSA* biofilm (Lu, et al. 2021). Endolysin *LysSA11*, at a concentration of 450 nM, reduced the optical density of the *S. aureus* culture after 30 minutes. However, the efficacy of *LysSA11* declined by 50% at temperatures of 4 °C or 65 °C (Chang, et al. 2017). Two other endolysins, *CHAPLysGH15* and *LysGH15*, that were isolated from *S. aureus*, showed a rapid antibacterial effect on *MSSA* and *MRSA* strains. Although they became inactive when exposed to heat treatment, *CHAPLysGH15* demonstrated high activity at pH 7.0–10.0, and *LysGH15* was active in high-salt environments. Therefore, they can be used in salty foods, as well as alkaline foods, including raw beef, pork, fish, and chicken, which are prone to contamination with *S. aureus* (Yan, et al. 2021). A well-studied, *S. aureus*-specific bacteriophage, Phage K, demonstrated a good inhibitory effect on *S. aureus* strains, including *MRSA*. Furthermore, when this phage was combined with essential oils, such as *a-pinene*, the inhibitory effect was greater than either the phage or the essential oil alone (Ghosh, et al. 2016). When Phage *SapYZU11* was applied at a multiplicity of infection (MOI) of 100, it resulted in the maximum reduction of *MRSA JCSC 4744* and *S. aureus* cocktail after 4 days, with reductions of 0.33 log CFU/mL and 0.29 log CFU/mL, respectively. These findings suggest that *SapYZU11* could be utilized as a biocontrol agent to effectively combat *S. aureus* contamination in the food industry (Wen, et al. 2023). Good results have been reported for combinations of phages and other antimicrobials, such as bacteriocins. For example, a combination of phage *SAP84* and a bacteriocin from *L. lactis CJNU* demonstrated significantly better *anti-S. aureus* activity

compared to each one alone *(Kim, et al. 2019)*. The synergistic inhibition of the combination of phage *SAP84* and bacteriocin against *S. aureus* caused a reduction of more than 5 log in viable counts, while the phage alone led to only about a 2 log cfu/mL reduction in *S. aureus* counts (Kim, et al. 2019). In another study, a lower concentration of endolysin *LysH5* was required in combination with subinhibitory concentrations of nisin to achieve complete inhibition of *S. aureus Sa9* (Arumugam, et al. 2019). After the treatment with 1 μM of recombinant *SAP8* endolysin, the initial *MRSA* count of 5.93 log CFU/mL was reduced to 3.64 log CFU/mL. In addition, the combination of 0.01 μM of recombinant *SAP8* endolysin and 18 IU/mL of nisin completely prevented the growth of *MRSA* (Hassan, et al. 2020). Also, the combination of bacteriophage endolysin *LysSA97* with *carvacrol* was found to significantly decrease the number of viable *S. aureus* cells (Chang, et al. 2017). When a combination of *S. aureus* phage (MOI 10) and 1% thyme oil was used, a greater reduction (87.22%) in *S. aureus* was achieved compared to using each treatment alone (Abdallah, et al. 2021). These examples indicate that phages can have a synergistic effect with other antibacterials. The effects of different bacteriophages and endolysins against *S. aureus*, including *MRSA*, have been reported in studies that are summarized in Table 2. Based on Table 2, the most effective phage compounds are *S. aureus* phage (containing *CHAPLysGH15* and *LysGH15)*, phage *SA11* endolysin *LysSA11*, endolysin *LysSA97* with *carvacrol*, and phage *endolysin LysH5* and *nisin*, respectively.

Table 2. Effect of bacteriophages and endolysins against *MRSA*

Bacteriophage			
Name	**Active Components**	**Effectiveness**	**References**
Endolysin *LysP108*	NR	MIC: 100 μg /mL	(Lu, et al. 2021)
Staphylococcus aureus bacteriophage	*CHAPLysGH15 and LysGH15*	*MRSA* was completely cleaved by 0.4 nmol/cm2 of *CHAPLysGH15*. 1.0 Log10 cfu/cm2 of *MRSA* declined after adding 0.4 nmol/cm2 of *LysGH15*	(Li, et al. 2011)

Bacteriophage			
Name	**Active Components**	**Effectiveness**	**References**
Endolysin *LysSA97* (an endolysin encoded by the bacteriophage *SA97*) with *carvacrol*	NR	The numbers of *S. aureus cells* were decreased by 0.8 ± 0.2 log cfu/mL and 1.0 ± 0.0 log cfu/mL at concentrations of 376 nm and 3.33 mm, respectively.	(Chang, et al. 2017)
EOCs (*a-pinene and 3-carene*) combined with two types of *S. aureus* bacteriophage, *phage K (ATCC 19685-B1)* and *phage 92 (ATCC 33741-B1)*	NR	Both phage of *S. aureus*-specific bacteriophage alone and EO *(a-pinene)* alone at 1.5 and 3.28 % yielded similar inhibition trends. However, with *phage K* and EOC (essential oil compounds) combinations, *phage K* with 3.28 % *a-pinene* inhibited *S. aureus* growth better than other combinations of EOCs and phage depending on the strain.	(Jaradat, et al. 2021)
Phage SA11 endolysin *LysSA11*	NR	The highest dose of Phage *SA11 endolysin LysSA11*(450 nM of endolysin) yielded a 50% reduction in optical density in less than 20 min and a 70% reduction within 30 min. *LysSA11* treatment (3.37μM, 1 h) reduced the number of *staphylococcal cells* in milk by about 2.53 log/mL	(Chang, et al. 2017)
Phage endolysin *LysH5* and *nisin*	NR	The MICs of *nisin* and *LysH5* were 3μg/mL and 50u/mL, respectively but in the presence of subinhibitory concentrations of *nisin*, a lower endolysin concentration was needed to fully inhibit *S. aureus Sa9*. These values implied up to a 64-fold and 16-fold reduction of the *nisin* and endolysin MICs, respectively, when used in combination.	(Arumugam, et al. 2019)

NR: not reported

Bacteriocins

According to studies, the growth of gram-positive pathogens, including *S. epidermidis*, *S. aureus*, and *MRSA*, was effectively inhibited by *NX371*, a novel class III bacteriocin gene. When *NX371* was added to milk, it moderately but significantly inhibited the growth of pathogens from day 1 to day 7, with reductions of 3.5 - 4.0 log in milk and 5.0 - 7.0 log in cheese, indicating its effectiveness as a food additive for controlling *S. aureus* in dairy products (Meng, et al. 2021). *Colicin* and *interocin* bacteriocins produced by *Escherichia coli* strains and *Enterococcus* species were found to have bactericidal effect against *MRSAs* and other *Staphylococcal* isolates, with complete bactericidal action achieved after 18-24 hours of incubation (Bajlan, et al. 2018). Bacteriocin produced by *Lactobacillus plantarum ZJ217 (plantaricin ZJ217)* was found to significantly decrease the colony forming units (Log10 CFU) of *S. aureus*, with viable cell counts decreasing from 6.5 ± 0.1 to 3.7 ± 0.04 log CFU/mL within 2 hours of incubation (Zhu, et al. 2015). Bacteriocin *KTH0-1S* produced by *Lactococcus lactis KTH0-1S* was found to significantly reduce the viable cell counts of *S. aureus* within 2 hours of incubation, with a higher proportion of dead cells compared to the control treatment (Saelao, et al. 2017). Bacteriocin *Paracin 54* produced by *Lactobacillus paracasei ZFM54*, was found to have a strong inhibitory effect on *Staphylococci*, with minimum inhibitory concentration values of 3.00 - 4.50 μg/mL (Zhu, et al. 2021). Bacteriocin producing *Pseudomonas aeruginosa TA6*, isolated from soil, was found to decrease the cell density of *S. aureus* rapidly, with cell lysis eventually occurring at concentrations of 100 AU/mL (Arumugam, et al. 2019). *Plantaricin 827*, produced by *Lactobacillus plantarum 163*, was found to quickly decrease *S. aureus* cells within 150 minutes of treatment with 64 μg/mL, and all *S. aureus* cells were destroyed within 90 minutes of treatment with 128 μg/mL. Moreover, *plantaricin 827* exhibited a certain preservation effect in skimmed milk and significantly extended the shelf life of skimmed milk (Zhao, et al. 2022). Bacteriocins produced by two strains, *Lactobacillus helveticus (BLh)* and *Lactobacillus plantarum (BLp*), had significant activity against *S. aureus* and *MRSA*. *L. helveticus (BLh)* was the most effective against *MRSA* after 18 to 24 hours of incubation at 37°C, while *L. plantarum (BLp)* had a similar effect against *MRSA* after 24 to 48 hours of incubation at 37°C under anaerobic conditions. The bacteriocin extracted from *L. plantarum (BLp)* was active even after passing through high temperature and pressure during sterilization, but the bacteriocin synthesized by *L. helveticus (BLh)* was more labile to heat (Hassan, et al. 2020). *Nisin*, a bacteriocin produced by the *Lactococcus lactis subsp. lactis* bacterium, exhibited

bacteriostatic activity against *MRSA* alone and had no effect against *S. aureus ATCC 25937*, while some strains of *Lactobacillus reuteri* produced *reuterin (β-hydroxypropionaldehyde)* under anaerobic conditions, which was considered to have bactericidal effects against *MRSA* and *S. aureus ATCC25937*. The combination of *nisin* at a concentration of 25.6 and *reuterin* at a concentration of 5.2 mg/mL exerted a bactericidal effect on *MRSA* and *S. aureus ATCC 25937* (Yehia, et al. 2022). Combinations of bacteriocins with other antimicrobials can increase their antibacterial efficacy. For instance, co-treatment of drinks with *enterocin* and phenolic compounds *(2NPOH)* resulted in the eradication of viable *staphylococci* after 24 hours (Burgos, et al. 2015). The effects of different types of bacteriocins against *S. aureus*, including *MRSA*, have been reported in studies that are summarized in Table 3. According to Table 3, the most effective bacteriocins against *S. aureus* are *Enterocin AS-48* with phenolic compounds or with *2NPOH*, Bacteriocin isolated from *Lactobacillus pentosus – Pentocin JL-1*, bacteriocin producing *Pseudomonas aeruginosa TA6*, and bacteriocin produced by *S. pasteuri RSP-1,* respectively.

Table 3. Effect of some bacteriocins against *MRSA*.

Bacteriocins			
Name	**Active Components**	**Effectiveness**	**References**
Bovine myeloid antimicrobial peptide (BMAP-28)	NR	20 mg/mL of *BMAP-28* could inhibit the growth of the two kinds of bacteria (*MRSA* and *MSSA*). MIC range (mg/mL): 5–20	(Takagi, et al. 2012)
Cell-free extracts of *Bifidobacterium*	*b1, b2, BL* and *BI*	MIC: 1.0 mg/mL	(AL-Saadi 2016)
Bacteriocin Produced by *B. cereus TSH5*	NR	MIC: 80 μg/mL	(Chauhan, et al. 2017)
Bacteriocin produced by *Staphylococcus pasteuri RSP-1 (S. pasteuri RSP-1)*	NR	MIC: 5 AU/mL	(Hong, et al. 2018)

Bacteriocins			
Name	**Active Components**	**Effectiveness**	**References**
Bacteriocin isolated from *Lactobacillus pentosus*	*Pentocin JL-1*	MIC: 7.5 µg/mL	(Jiang, et al. 2017)
Enterocin *AS-48* with phenolic compounds or with *2NPOH*	NR	No viable *staphylococci* were detected after 24 h incubation with the combination of *enterocin AS-48* and *2NPOH*	(Murray, et al. 2021)
Bacteriocin from *Lactococcus lactis KU24*	*Bacteriocin KU24*	*S. aureus ATCC 33591* was inhibited *by bacteriocin KU24* at 2 Log cfu/mL after 10 h of incubation. MIC: 400 to 800 AU/mL	(Lee, et al. 2013)
Bacteriocin producing *Pseudomonas aeruginosa TA6*	NR	MIC: 50 AU/mL the cell density of *S. aureus* decreased rapidly, and cell lysis occurred at 100 AU/mL concentrations	(Zhou, et al. 2017)
Bacteriocin producing *Lactobacillus acidophilus*	*bacteriocin gene NX371*	MIC90 was ranged from 20 to 160 µg/mL	(Meng, et al. 2021)
Bacteriocins produced by *Escherichia coli* and *Enterococcus species*	*Colicin and interocin*	The incubation times for complete bactericidal action were 18-24h.	(Bajlan, et al. 2018)
Bacteriocin produced of *Lactococcus lactis KTH0-1S*	*Bacteriocin KTH0-1S*	The proportion of dead cells was significantly higher since viable cell counts decreased from 6.5±0.1 to 3.7±0.04 log CFU/mL within 2 h of incubation	(Saelao, et al. 2017)
Bacteriocin produced of *Lactobacillus paracasei ZFM54*	*Bacteriocin Paracin 54*	MIC: 3.50 µg/mL	(Zhu, et al. 2021)

Bacteriocins			
Name	**Active Components**	**Effectiveness**	**References**
Bacteriocin producing from *Pseudomonas aeruginosa TA6*	NR	Maximum bacteriocin activity (100AU/mL) was observed at 37 °C in 24 h time duration.	(Arumugam, et al. 2019)
Bacteriocin produced by *Lactobacillus plantarum 163*	*Plantaricin 827*	MIC: 64 μg/mL.	(Zhao, et al. 2022)
Bacteriocin produced by Lactobacillus helveticus and Lactobacillus plantarum		L. helveticus showed the activity against MRSA after 18 to 24 hours of incubation at 37°C. In comparison, L. plantarum showed similar activity against MRSA after 24 to 48 hours of incubation at 37°C under anaerobic conditions.	(Hassan, et al. 2020)
Bacteriocin produced by Lactococcus lactis subsp. lactis. and Lactobacillus reuteri	*Nisin and reuterin*	MIC of nisin: 51.2 mg/ mL, MIC of reuterin: 5.2mg/mL MBC of nisin: 5 mg/mL, MBC for reuterin: 5 mg/mL	(Yehia, et al. 2022)

NR: not reported

Probiotics

The most common probiotics are *lactic acid bacteria (LAB)* strains, and they are considered safe. *LAB* can produce bactericidal bioactive peptides and enzymes that have antibacterial and antibiofilm effects (Hermawati, et al. 2016). For instance, *Lactobacillus* can inhibit *Staphylococcal cells*, including *MRSA* (Hermawati, et al. 2016). Several probiotics such as *Lactobacillus plantarum* (Lee, et al. 2021, Afshari, et al. 2022), *Lactobacillus acidophilus*, *Lactobacillus casei* (Hermawati, et al. 2016), *Streptomyces griseus*, *Lactococcus lactis*, *Streptococcus*, *Leuconostoc*, and *Pediococcus* (Li, et al. 2011) have demonstrated

inhibitory effects on *S. aureus* strains, including *MRSA*. Table 4 shows the effects of different probiotics on *MRSA*. According to Table 4, the most effective probiotics were *Streptomyces griseus*, *Pediococcus acidilactici strains A11 and C12, Lactococcus lactis*, and *Lactobionic acid*, respectively.

Table 4. Effect of some probiotics against MRSA

Probiotic			
Name	**Active Components**	**Effectiveness**	**References**
Lactobacillus acidophilus and probiotic *Lactobacillus casei*	NR	MIC: 3.12% for *Lactobacillus acidophilus* and 2% *Lactobacillus casei*	(Karska-Wysocki, et al. 2010)
Probiotic *Lactobacillus plantarum KU200656*	NR	MIC :12.5%	(Lee, et al. 2021)
Pseudomonas fluorescens	*Pseudomonic acids and Mupirocin*	MIC of 8-256 µg/mL for low level resistance and 512 µg/mL for high level resistance	(Prastiyanto, et al. 2020)
Streptomyces griseus	*Treptose, streptydine, and Nmethyl- L –glycosamine and Streptomycin*	MIC: 1.56-6.25 µg/mL	(Prastiyanto, et al. 2020)
Lactococcus lactis	*Lnathionine (Lan), methyllanthionine (MeLan), didehydroalanine (Dha) and didehydroaminobutyric acid (Dhb) and Nisin*	MIC: 1.5 to > 1.6 mg/L	(Prastiyanto, et al. 2020)
Streptococcus, Leuconostoc, Lactobacillus, and *Pediococcus*	*Diacetyl*	MIC: 1.00 µg/mL	(Prastiyanto, et al. 2020)
Pediococcus acidilactici strains A11 and C12	NR	MIC: 25%, MBC: 12.5%	(Lestari, et al. 2019)

NR: not reported

DISCUSSION

Essential oils

Several possible mechanisms have been proposed for antibacterial activity of essential oils. Table 4 shows the mechanisms of action of reported EOs. Essential oils can inhibit enzymes and compounds that are needed for the growth of *MRSA* (Eom, et al. 2014; Burris, et al. 2015). For example, *cinnamaldehyde* in cinnamon oil inhibits *N-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL)* and *AI-2* (Salem 2017) while allicin liquid can inhibit sulfhydryl enzymes, thereby inhibiting DNA and protein synthesis (Cutler, et al 2004; Li, et al. 2011). Some EOs can cause the release of intracellular components (Eom, et al. 2014; Jaradat, et al. 2021), such as brown algae, which lead to the release of intracellular components via phlorofucofuroeckol (PFF) (Eom, et al. 2014). Inhibition of enzymes by EOs can occur through participation in electron transport with the cell components and binding to bacterial adhesions and cell walls (Ifesan, et al. 2009). *Aloysia citriodora* EO can participate in the lipophilic lipids of the mitochondria and cytoplasmic membrane due to their lipophilic ability (Jaradat, et al. 2021). The lipophilic characteristics of EOs make them capable of easily penetrating the bacterial cell (Prastiyanto, et al. 2020). For example, *terpenoids* in mushroom *Pleurotus flabellatus* have this ability and can interfere with protein synthesis and DNA replication (Prastiyanto, et al. 2020). Additionally, some EOs such as EOs derived from *Chuzhou chrysanthemum* and *clove buds* increase the permeability of the cell membrane, resulting in the leakage of intracellular essential substances such as electrolytes, protein, and nucleic acids (Xu, et al. 2016; Cui, et al. 2018). Many EOs such as *carvacrol* are safe to apply in foods as a natural food preservative and are 'Generally Recognized as Safe' (GRAS) by the US Food and Drug Administration (FDA) (Chang, et al. 2017). EOs can have an enhanced effect when they are used at high concentrations (Higginbotham, et al. 2014). Furthermore, if they are applied in processed foods such as hot dogs, chemicals like potassium lactate, sodium lactate, sodium diacetate, and sodium nitrite, they can improve the antimicrobial activity of the Eos (Higginbotham, et al. 2014). Black seed *(Nigella sativa*) is a type of medicinal herb that contains bioactive substances of medical importance. The GC-MS analysis of *N. sativa* shows that it contains five essential compounds, all of which are a unique mix of organic compounds and alkaloids that possess high biological activity, such as *Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL, and Pentane, 2-cyclopropyl* (Abdullah, et al. 2021). *Litsea cubeba* essential oil *(LC-EO)* can cause *MRSA* cell rupture, which results in the leakage of cellular content and ultimately

leads to the bacteria's death. LC-EO treatment decreases the activity of four ATPases, including the Na+/K+ ATPase, Ca2+/ Mg2+ATPase, Ca2+ATPase, and Mg2+ATPase (Hu, et al. 2019). *Chinese Red Propolis* is rich in *pinobanksin* and *pinobanksin-3-acetate*, and its antibacterial activity may be the result of the synergistic effect of polyphenols (Zhang, et al. 2022). *Carum carvi L.* disrupts *MRSA* biofilm and amino acid metabolism, and it also hinders DNA and RNA synthesis (Liu, et al. 2023). *Psoralea corylifolia* seed ethanol extract *(PCEE)* is composed of *phenol*, *hydrazine*, *aldehyde*, and *ketone*, which can destroy the cell structure and reduce enzymes, ultimately killing bacteria (Li, et al. 2019). *Backhousia citriodora* Essential Oil *(BCEO)* contains large amounts of *oxygenated monoterpenes*, which disrupt the microbial cytoplasmic wall, improve cell permeability, and lead to cell death (Lim, et al. 2022). Oregano essential oil (OEO) and cinnamon essential oil (CEO) increase cell permeability and cause leakage of intracellular constituents, leading to the disruption of the cell respiration system and microbial enzyme system (Debiagi, et al. 2020). *L. micromera* and *P. amboinicus* essential oils contain *monoterpenes* such as *carvacrol*, *γ-terpinene*, and *β-cymene*, which are responsible for their antibacterial activity against *Staphylococcus species* including *MRSA* (Bugayong, et al. 2019). *Elettaria cardamomum* essential oil blurs the surface barrier of the cell wall, altering the structure of the cells, and causing bacterial mortality (Jha, et al. 2022). The combination of EOs can enhance the efficacy of their antibacterial activity. For instance, when *carvacrol* and *thymol* are combined with organic acids, a significant reduction in the number of *S. aureus* is observed on food samples. On one hand, these EOs disrupt the bacterial cell membrane and make the bacteria more susceptible to the acidic environment. On the other hand, organic acids enhance the hydrophobicity of EOs and make the EOs bind better to hydrophobic regions of the membrane proteins (de Oliveira, et al. 2010). Thyme oil, when combined with lytic *S. aureus* phage, is a promising biocontrol agent and antimicrobial alternative in the food industry to control and reduce *MRSA* or other *antibiotic-resistant S. aureus* contamination in food (Abdallah, et al. 2021). Some plant extracts can increase the effectiveness of antibiotics against *MRSA*. For instance, *ursolic acid 3-O-α-L-arabinopyranoside (URS)* from the leaves of *Acanthopanax henryi* (Oliv.) can enhance the *anti-MRSA* effect of oxacillin (Zhou, et al. 2017).

Bacteriophages

Bacteriophages encode peptidoglycan hydrolases, known as endolysins or lysins, which lyse bacterial cells by targeting their cell wall, particularly in Gram-positive bacteria, due to their naturally exposed peptidoglycan layer (Murray,

et al. 2021). Bacterial death by endolysins is in accordance with the typical phenomenon of osmotic-mediated cell lysis, which occurs in Gram-positive bacteria following a phage attack (Lu, et al. 2021). For instance, *LysP108* causes disintegration of the *MRSA* cell wall (Lu, et al. 2021). It has been reported that a combination of endolysin *LysSA97* with *carvacrol* can cleave bacterial peptidoglycan layers and destroy the structure of the cell wall (Chang, et al. 2017). Combining endolysins with antibiotics causes better accessibility of antibiotics to *MRSA* cells through initial lysing of the biofilm by endomysia (Linden, et al. 2015). Combining endolysins with bacteriocins can result in a higher sensitivity of *S. aureus* cells to these antibacterials. The mechanism might be attributed to the prevention of peptidoglycan breaks produced by endolysins from contraction (Arumugam, et al. 2019). Moreover, bacteriocins can cause a partial activation of autolysins that allows for better activity of the endolysin (Arumugam, et al. 2019). The combination of synthetic *SAP8 endolysin* and *nisin* can effectively restrain various types of Gram-positive bacteria by creating openings in the bacterial cell membrane and blocking the production of cell walls (Kim, et al. 2022).

Bacteriocin

Bacteriocins can cause damage to the cell wall or induce cell lysis (Lee, et al. 2013). They target the cytoplasmic membrane of bacterial cells and inhibit the proton motive force (PMF), leading to inhibition of protein or nucleic acid production (Lestari, et al. 2019). The *anti-MRSA* activity of bacteriocins is mainly due to the generation of organic acids such as *lactic acid* and *acetic acid*. These acids enter bacterial cells and interfere with essential metabolic processes (AL-Saadi 2016). Bacteriocins against *MRSA* can change the cell surface from smooth to rough. Therefore, the suggested mechanism is related to the bacterial cell membrane (Takagi, et al. 2012). Several bacteriocins have been reported to have *anti-MRSA* activity, leading to the disruption in the integrity and uniformity of *MRSA* (Zhu, et al. 2015; Jiang, et al. 201; Taggar, et al. 2021). Through bioinformatic analysis of *Lactobacillus acidophilus*, a new class III bacteriocin gene called *NX371* was discovered, which demonstrated high antimicrobial activity across a wide range of pH values (3.0-8.0). This bacteriocin was able to disrupt the cell wall of gram-positive bacteria and induce membrane leakage in gram-negative bacteria, leading to separation of the cell wall and membrane (Meng, et al. 2021). Other bacteriocins such as *colicins* and *enterocins* also exhibit antibacterial effect against Gram-positive bacteria, with *colicins* acting as transmembrane proteins that depolarize the cytoplasmic

membrane and kill cells by producing pores or acting as a nuclease to chop up DNA or RNA (Bajlan, et al. 2018). Another example is *plantaricin ZJ217*, a novel bacteriocin produced by *Lactobacillus plantarum ZJ217*, which was inhibitory effect against a variety of gram-positive and gram-negative bacteria by forming pores in cells (Zhu, et al. 2015). Similarly, bacteriocin *KTH0-1S* produced by *Lactococcus lactis KTH0-1S* acted on sensitive cells by forming pores in membranes, leading to cell death due to the loss of essential intracellular substances (Saelao, et al. 2017). *Paracin 54*, a bacteriocin produced by *Lactobacillus paracasei ZFM54*, also formed pores in the cell membrane of *MRSA*, which disrupted the balance of ions inside and outside the membrane and led to the dissipation of proton driving force, inhibiting the synthesis of intracellular ATP and causing the disorder of intracellular energy metabolism (Zhu, et al. 2021). Another bacteriocin, *plantaricin 827*, produced by *Lactobacillus plantarum 163*, had antibacterial effects against *MRSA* by increasing the cell membrane permeability and integrity, resulting in the leakage of K+ and changes in cell morphology, inhibiting biofilm formation, and interacting with genomic DNA minor groove in AT-rich regions (Zhao, et al. 2022). The combination of *nisin* produced by *Lactococcus lactis subsp. lactis* and *reuterin* produced by *Lactobacillus reuteri* also disrupted membranes by forming pores, inhibiting energy production and biosynthesis of proteins and nucleic acids (Yehia, et al. 2022). Despite the fact that they are proteins, some bacteriocins can remain stable in harsh environmental conditions. For instance, *Paracin 54* retained 93.7% of its activity after treatment with lysozyme, indicating its potential for use in food preservation. Furthermore, *Paracin 54* maintained its inhibitory activity against *MRSA* at different temperatures, suggesting its potential use in pasteurized products (Zhu, et al. 2021). *Plantaricin* 827 also exhibited antibacterial activity at pH 7.0, while *plantaricin ZJ217* was stable at pH 2.0 to 6.0 but lost activity at pH 10.0 (Zhu, et al. 2015).

Probiotics

The major effects of probiotics include modulation of the immune system, inhibition of pathogen adhesion to epithelial cells, and generation of antimicrobial compounds (AL-Saadi 2016; Lee, et al. 2021). Antimicrobial compounds produced by probiotics can also demonstrate anti-adhesion ability (Lee, et al. 2021). These antimicrobial components include organic acids, oxygen catabolites, and proteinaceous compounds (Lee, et al. 2021). These components can inhibit the growth of *MRSA* cells in food products (Karska-Wysocki, et al. 2010). For instance, *Lactococcus lactis* can generate antibacte-

rial agents, including *didehydroaminobutyric acid (Dhb)* and *didehydroalanine (Dha)*, *methyllanthionine (MeLan)*, *Lanthionine (Lan)*, and bacteriocin *(Nisin)*. These substances can disrupt the uptake of amino acids by *S. aureus* cells and suppress the production of the cell wall. In addition, some metabolites will be released, leading to cell death (Li, et al. 2011). Generally, *LBA* can cause alkaline phosphatase leakage from *MRSA cells* to the extracellular medium, and in this way, they prevent the formation of biofilms (Kang, et al. 2020). A list of *anti-S. aureus* agents and their modes of action is in Table 5.

Table 5. Mechanisms of action of anti-MRSA EOs, bacteriophages, bacteriocins, and probiotics.

Name	Active Components	Mechanisms	References
Essential oils			
Essential oils: Cinnamon oil, Thyme oil and Lemongrass oil	*Cinnamaldehyde, eugenol, Alpha and beta-citral, mycrene*	These can inhibit *N-3-oxohexanoyl- Lhomoserine lactone (3- oxo-C6-HSL)* and *AI-2,* and certain enzymes needed for the growth of *MRSA*.	(Salem 2017)
Liposome containing cinnamon oil	NR	The damage of bacterial cell membrane is by their effect on morphology, structure, function, modification in the transport of nutrients, membrane disruption, extensive leakages from the bacterial cells leading to cell death.	(Cui, et al. 2016)
Indian Spices	*Syzygium aromaticum (CLV) and Cinnamomum zeylanicum (CIN) and Cuminum cyminum (CMN)*	These can affect the synthesis of the peptidoglycan layer of the cell wall and the mode of action of the spice extracts is cell wall related.	(Mandal, et al. 2011)

Name	Active Components	Mechanisms	References
Essential oils			
Camellia sinensis and Azadirachta indica leaves	*catechin*	The *catechin* has direct effects on the destruction of the bacterial cell membrane by binding with the lipid bilayer.	(Zihadi, et al. 2019)
Allicin	NR	Inhibit the acetyl coA forming system, to inhibit DNA and protein synthesis, and to target RNA polymerase.	(Cutler, et al 2004)
Cinnamomum osmophloeum leaf essential oils	*cinnamaldehyde*	NR	(Chang, et al. 2001)
Thyme (Tymus vulgaris) and Oregano (Origanum vulgare) essential oils	*Thymol* from *Thyme* and *Carvacrol* from *Oregano*	Phosphate ion leakage.	(Boskovic, et al. 2015)
PFF (phlorofucofuroeckol, a marine-derived polyphenol found in brown algae)	NR	Interfering with cell wall synthesis and the cell membrane and agents change membrane function and permeability, leading to cell damage or death.	(Eom, et al. 2014)

Name	Active Components	Mechanisms	References
Essential oils			
URS (ursolic acid 3-O-α-L arabinopyranoside was isolated from the leaves of A. henryi (Oliv) with oxacillin	Q	Deformation of bacterial cells. Cell membrane disintegration, cell lysis and release of cytoplasmic contents.	(Yan, et al. 2021)
Pink oyster mushroom Pleurotus flabellatus	The terpenoid compound group	These penetrate the bacterial cell and may interfere with protein synthesis and DNA replication.	(Ghosh, et al. 2016)
Amomum villosum Lour	Bornyl acetate	Leakage of intracellular macromolecular substances.	(Tang, et al. 2020)
Bulb Eleutherine Americana	Naphthoquinone	Inhibits electron transport with the cell components. They also can bind to bacterial adhesions and complex with cell wall, thus inactivating enzymes.	(Ifesan, et al. 2009)
Aloysia citriodora essential oils EOs from Baqa al-Gharbiyye and Umm al-Fahm	lipophilic structures like α- citral and α-curcumene	Their lipophilic ability to partition in the lipophilic lipids of the mitochondria and cytoplasmic membrane. They could also disturb the structures, resulting in leakage of bacterial cell contents.	(Arumugam, et al. 2019)

Name	Active Components	Mechanisms	References
Essential oils			
Garlic	Allicin (allyl 2-propenethiosulphinate)	1) The primary mechanism of allicin centers on its ability to inhibit sulfhydryl enzymes common for pathogenic bacteria. 2) Inhibiting enzymes associated with DNA and protein synthesis and limiting RNA polymerase and alcohol dehydrogenase activities.	(Prastiyanto, et al. 2020)
flavonoids from licorice	glabrol, licochalcone A, licochalcone C, and licochalcone E	Disruption of membrane permeability. The binding of these to the cell wall or the cytoplasmic membrane is important for their action on the bacterial membrane.	(Burgos, et al. 2015)
Clove buds	Eugenol	The permeability of bacterial membrane would be increased, which caused the leakage of intracellular ingredient, especially losses of electrolytes including K+, Ca2+, Na+, as well as cell constituents such as protein, nucleic acids, and some essential molecules.	(Xu, et al. 2016)
Chuzhou chrysanthemum	B-Eudesmene, L-Borneol, Camphor	Disruption of the cell membrane and leakage of DNA, protein and ATP to the bulk solution.	(Cui, et al. 2018)

Name	Active Components	Mechanisms	References
Essential oils			
Syzygium antisepticum plant	b-caryophyllene	Membrane-disrupting effect was observed	(Yuan, et al 2018)
Korean soybean fermented product doenjang	Methanolic	Inhibits the respiratory metabolism and protein synthesis of the bacteria and prevents nucleic acid synthesis. Thus, it affects the integrity of the cell wall and membrane.	(Lalouckova, et al. 2021)
Bisdemethoxycurcumin with three antibiotics (gentamicin, ampicillin, and oxacillin)	NR	The polyphenol structure can destroy the cell wall of bacteria and thus increases the efficiency of antibiotics entering the cell.	(Wang, et al. 2020)
Thymol and carvacrol with organic acids (lactic acid)	NR	Phenolic compounds can damage cellular membrane changing their structure and function and causing it to become more susceptible to acid environments. On the other hand, at low pH the molecules of thymol and carvacrol are mostly dissociated, more hydrophobic, and bind better to hydrophobic regions of the membrane proteins resulting in better partition into the lipid phase of the bacterial membrane.	(de Oliveira, et al. 2010)

Name	Active Components	Mechanisms	References
Essential oils			
Litsea cubeba essential oil	β- Citral and α-Citral	The LC-EO could lead to the rupture of MRSA cells and the loss of cellular contents and eventually to the death of bacteria.	(Hu, et al. 2019)
Red Propolis	Pinobanksin, Pinobanksin-3-acetate	The mechanism of action of RPE due to loss of membrane integrity.	(Zhang, et al. 2022)
Ethanol Extracts of Psoralea corylifolia Seeds	phenol, hydrazine, aldehyde, and ketone	PCEE could change the membrane integrity of MRSA, releasing nucleic acids and proteins, resulting in bacterial death.	(Li, et al. 2019)
Manuka EO was extracted from manuka leaves	Sesquiterpenes	MIC: 0.233 mg/mL, MBC: 0.466 mg/mL	(Pedonese, et al. 2022)
Essential Oils from Elettaria Cardamomum fruit capsules	Monoterpenes and sesquiterpenes	Elettaria cardamomum EO damages the biofilm barrier, causing the bacteria to lose metabolic activity.	(Jha, et al. 2022)
Bacteriophages			
Phage Endolysin *LysP108*	NR	*LysP108* disintegrated the cell wall of *MRSA*.	(Lu, et al. 2021)

Name	Active Components	Mechanisms	References
Bacteriophages			
Bacteriophage *endolysin plygrcs*	*PlyGRCS*	The endolysin *plygrcs* would provide the initial disturbance to the biofilm structure.	(Linden, et al. 2015)
Carvacrol and lyssa97	NR	*LysSA97* cleaving bacterial peptidoglycan layers is likely to render the cell wall structure less rigid so that *carvacrol* may more readily reach the cytoplasmic membrane of S. *aureus*.	(Chang, et al. 2017)
Phage endolysin lysh5 and nisin	NR	*LysH5 activity might be increased by the permeabilization of the cytoplasmic membrane by nisin. Also, partial activation of autolysins by nisin may occur and facilitate LysH5 activity.*	(García, et al. 2010)
SAP8 endolysin	NR	*Forms pores in bacterial cytoplasmic membrane and inhibits cell wall synthesis*	(Kim, et al. 2022)
Bacteriocins:			
Bovine myeloid antimicrobial peptide (BMAP-28)	NR	1) Cell wall permeation is made by the activity of *BMAP-28* as it is diffusing inside the bacteria. 2) Bacterial smooth surface somehow changes into a rough surface by the activity of *BMAP-28*. 3) *BMAP-28* can break *MRSA* cell membranes.	(Takagi, et al. 2012)

Name	Active Components	Mechanisms	References
Bacteriocins			
Pediococcus acidilactici strains A11 and C12	NR	The initial bacteriocin reaction is to damage membrane permeability and eliminate proton motive force *(PMF)* thereby inhibiting energy production and biosynthesis of proteins or nucleic acids. 2) bacteriocin molecules are in direct contact with cell membranes, this contact process is able to disrupt membrane potential in the destabilizing cytoplasmic membranes so that cells become less strong, and membrane instability is capable of producing holes in cell membrane through the process of interference with PMF (Proton Motive Force).	(Lestari, et al. 2019)
Cell-free extract of *Bifidobacterium Species of LAB*	*b1, b2, BL* and *BI*	The acids produced by *LAB* enter the sensitive bacterial cells and interfere with the necessary metabolic process such as substrate translocation and oxidative phosphorylation, which leads to a decrease in the internal pH of bacterial cells.	(AL-Saadi 2016)

Name	Active Components	Mechanisms	References
Bacteriocins			
bacteriocin produced by *Staphylococcus pasteuri RSP-1 (S. pasteuri RSP-1)*	*Pasteuricin*	*Pasteuricin* rapidly damaged the membrane of viable cells.	(Hong, et al. 2018)
Bacteriocin isolated from *Lactobacillus pentosus*	*Pentocin JL-1*	*Pentocin JL-1* targets the cell membrane of *MRSA GIM 1.771*, causing a loss of PMF in only a few minutes, and that has a dramatic impact on the structure and integrity of the *MRSA* cell and finally leads to cell death.	(Jiang, et al. 2017)
Bacteriocin from *Lactococcus lactis KU24*	*Bacteriocin KU24*	The bacteriocin *KU24* damages the cell wall or induces cell lysis and has an impact on the bacterial cytoplasmic membrane.	(Lee, et al. 2013)
Bacteriocin from Lactobacillus plantarum ZJ217	*NR*	Bacteriocin produced by Lactobacillus plantarum can cause the formation of pores on bacterial cells and releasing ATP, and bacterial death	(Zhu, et al. 2015)
Bacteriocin isolated from the natural inhabitant of Allium cepa	*Peptide-Ba49*	Peptide-Ba49 can result in the rupturing and uniformity of MRSA.	(Taggar, et al. 2021)

Name	Active Components	Mechanisms	References
Bacteriocins			
Bacteriocin producing Lactobacillus acidophilus	*Bacteriocin gene NX371*	The leakage of intracellular ATP, disrupt the cell wall, and induce membrane leakage.	(Meng, et al. 2021)
Bacteriocin produced by Escherichia coli strains and Enterococcus species	*Colicins and Enterocin*	These depolarize the cytoplasmic membrane, leading to dissipation of cellular energy and killing domain may produce a pore in the target cell membrane, or act as a nuclease to chop up the DNA or RNA of the target cell	(Bajlan, et al. 2018)
Bacteriocin produced by Lactobacillus plantarum ZJ217	*Plantaricin ZJ217*	The Plantaricin ZJ217 had activity against biofilm cells of MRSA by forming pores to release ATP.	(Zhu, et al. 2015)
Bacteriocin produced of Lactococcus lactis KTH0-1S	*Bacteriocin KTH0-1S*	The KTH0-1S can have an impact on sensitive cells, causing pores formation in the membrane, resulting in cell death due to loss of essential intracellular substances	(Saelao, et al. 2017)
Bacteriocin produced by Lactobacillus paracasei ZFM54	*Bacteriocin Paracin 54*	The treatment with Paracin 54 enhanced the permeability of the cell membrane, damaged the cell membrane, and led to electrolyte outflow.	(Zhu, et al. 2021)

Name	Active Components	Mechanisms	References
Bacteriocins			
Bacteriocin produced by Lactobacillus plantarum 163	*Plantaricin 827*	Its antibacterial mechanism increased the cell membrane permeability and integrity, resulting in the leakage of K+ and changes in cell morphology.	(Zhao, et al. 2022)
Bacteriocin produced by Lactococcus lactis subsp. lactis. and Lactobacillus reuteri	*Nisin and reuterin*	The combination of nisin and reuterin may change the permeability of the outer membrane and cause a lethal effect.	(Yehia, et al. 2022)
Probiotics			
Lactobacillus plantarum KU200656	NR	It can inhibit the adherence of pathogens by competing for nutrition and host intestinal cell-binding sites, e.g., receptor exclusion. bolites (e.g. hydrogen peroxide), and proteinaceous compounds.	(Lee, et al. 2021)
Lactobacillus acidophilus and *Lactobacillus casei*	NR	They produce antimicrobial components that can inhibit the growth and eliminate of the *MRSA* cells.	(Karska-Wysocki, et al. 2010)

NR: not reported.

CONCLUSION

According to our review, the most effective EO against *MRSA* was a liposome containing cinnamon oil, which resulted in a significant decrease in *MRSA* populations. Additionally, essential oils from *Cinnamomum zeylanicum*, *Syzygium aromaticum*, *Cuminum cyminum*, allicin, *glabrol, clove buds,* and *backhousia citriodora* have shown significant effectiveness against *MRSA*. Another potential solution against *MRSA* is the use of bacteriophages. Based on our review, promising phage compounds include *S. aureus* phage containing *CHAPLysGH15* and *LysGH15* and *phage SA11 endolysin*. Bacteriocins have also shown promise in combatting *MRSA*. Bacteriocins such as *Enterocin AS-48, Pentocin JL-1*, bacteriocin-producing *Pseudomonas aeruginosa TA6*, and bacteriocin produced by *S. pasteuri RSP-1* were effective against *S. aureus*. Probiotics have also shown antimicrobial properties against *MRSA*. *Streptomyces griseus*, *Pediococcus acidilactici* strains *A11 and C12, Lactococcus lactis*, and *Lactobionic acid* are among the most effective probiotics against *MRSA*. In the fight against *MRSA*, the combination of above-mentioned antibacterials has also shown promising results. These natural compounds and microorganisms possess unique mechanisms of action that can effectively target and eliminate *MRSA* cells. Furthermore, their use in combination with other antimicrobial agents including chemicals can enhance their efficacy, providing a multi-hurdle approach in combatting antibiotic-resistant bacteria. However, in food matrices, the results might be different from in vitro experiments because natural compounds can interact with food compounds like proteins and lipids, potentially reducing the availability of the natural compound as an antimicrobial agent. Additionally, processing steps can diminish the antimicrobial activity of such compounds. Furthermore, before the application of natural antimicrobials in food products, health and safety risks associated with them should be thoroughly assessed. The impact of EOs on the organoleptic characteristics of food should also be taken into account, as they may have negative effects. Nevertheless, the application of nanotechnology can mitigate these effects.

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Author's Contribution:

Z.A. made contributions to conception and design of the study, was involved in data collection and drafting the manuscript. G.S. revised the manuscript critically and together with Z.A., M.H., and A.A. prepared the final draft of the manuscript etc. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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POTENTIAL OF DIFFERENT MYCOTOXIN ADSORBENTS UNDER *IN VITRO* CONDITIONS

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Abstract

Mycotoxins are a large and chemically diverse group of toxic secondary metabolites. Regarding their prevalence in animal feed and the effect on animal health, the biggest problems in terms of safety and economic losses are caused by aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone. Adsorbents are substances that are added to food contaminated with mycotoxins, in order to bind them in the gastrointestinal tract and thereby prevent or reduce their effect. The aim of this study was to examine the possibility of using pyrophyllite as a mineral adsorbent, as well as preparations made of ground peach pits of different particle sizes as organic adsorbents, for adsorption of deoxynivalenol and ochratoxin A. Mycotoxin adsorption experiments were performed *in vitro* in electrolyte solutions at pH 3 and 7. The adsorption efficiency of the adsorbent was expressed as adsorption index. Pyrophyllite had adsorption index of 13.47% for ochratoxin A at pH 3, while at pH 7, as well as for deoxynivalenol, the same mycotoxin produced a negligible degree of adsorption. Ground peach stones (of larger diameter, d = 0.1 mm) had considerable adsorption rates for ochratoxin A at pH 3 (34.41%) and deoxynivalenol at pH 7 (18.57%). The values were similar for smaller diameter (d < 0.1 mm) for ochratoxin A at pH 3 (42.71%) and deoxynivalenol at pH 7 (20.11%). The obtained results suggest that the potential of the preparation of ground peach stones for the adsorption of tested mycotoxins is higher compared to the potential of pyrophyllite, but there are differences in their efficiency depending on the pH value of the adsorption environment.

Key words: ochratoxin A, deoxynivalenol, adsorbents, pyrophyllite, peach stones

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POTENCIJAL RAZLIČITIH ADSORBENATA MIKOTOKSINA U *IN VITRO* USLOVIMA

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Kratak sadržaj

Mikotoksini su relativno velika i hemijski raznolika grupa toksičnih sekundarnih metabolita. Uzimajući u obzir prevalencu u hrani za životinje i efekat na zdravlje životinja, najveće bezbednosne i ekonomske probleme predstavljaju aflatoksini, fumonizini, ohratoksini, trihoteceni i zearalenon. Adsorbenti predstavljaju materije koje se dodaju hrani kontaminiranoj mikotoksinima, kako bi ih u gastrointestinalnim traktu vezivali i time sprečili ili umanjili njihovo dejstvo. Cilj ovog rada je da se ispita mogućnost primene pirofilita kao predstavnika mineralnih, kao i preparata mlevenih koštica breskve različitih veličina čestica kao predstavnika organskih adsorbenata za adsorpciju deoksinivalenola i ohratoksina A. Eksperimenti adsorpcije mikotoksina su izvođeni *in vitro* u rastvorima elektrolita na pH 3 i 7. Efikasnost adsorpcije adsorbenta je izražena kao indeks adsorpcije. Rezultati su ukazali da pirofilit ima vrednost indeksa adsorpcije od 13,47% za ohratoksin A pri pH 3, dok je za isti mikotoksin pri pH 7, kao i za deoksinivalenol, utvrđen zanemarljiv nivo adsorpcije. Mlevene koštice breskve većeg dijametra (d = 0,1 mm) su pokazale odgovarajuće indekse adsorpcije za ohratoksin A pri pH 3 (34,41%) i deoksinivalenol pri pH 7 (18,57%), dok su mlevene koštice breskve manjeg dijametra (d < 0,1 mm) pokazale slične vrednosti indeksa adsorpcije za ohratoksin A pri pH 3 (42,71%) i za deoksinivalenol pri pH 7 (20,11%). Iz dobijenih rezultata se može zaključiti da je potencijal preparata mlevenih koštica breskve za adsorpciju ispitanih mikotoksina veći u odnosu na potencijal pirofilita, ali da postoje razlike u njihovoj efikasnosti u zavisnosti od pH vrednosti sredine u kojoj se adsorpcija odvija.

Ključne reči: ohratoksin A, deoksinivalenol, adsorbenti, pirofilit, koštice breskve

INTRODUCTION

Agriculture is becoming increasingly important due to the rise in world population and urbanization. This implies a rapid growth and boost of livestock production, resulting in the safety of animal feed becoming even more significant considering the potential threat of hazards reaching the human food chain. Among the undesirable substances, according to Directive 2002/32/EC (European Commission, 2002), mycotoxins are considered one of the greatest hazards in feed raw materials (Santos Pereira et al., 2019). Among other mycotoxins, deoxynivalenol (DON) and ochratoxin A (OTA) certainly represent the most important causes of diseases in animals. Namely, global economic losses caused by DON are in the range of about one billion dollars per year. The Food and Agriculture Organization and the World Health Organization identified it as one of the most dangerous food contaminants as early as 1973 (Neme and Mohammed, 2017). This mycotoxin mainly contaminates agricultural crops such as corn, wheat, and barley, and has immunogenic, carcinogenic, and teratogenic effects in animals. It is also detected in animal products such as meat, eggs, and milk, and poses a danger to human health (Yao and Long, 2020). OTA causes great economic losses to animal husbandry, as the intake of contaminated feed can significantly impair animal health and safety of animal products (Battacone et al., 2010). It can be found in a wide range of agricultural and livestock products, and in processed foods as well, while human exposure to this mycotoxin is mainly attributed to contaminated grains (Abrunhosa et al., 2010). Juodeikiene et al. (2012) stated three possible solutions for avoiding the harmful effect of mycotoxin contamination of both food and feed. They include the following: prevention of contamination, decontamination of mycotoxin-containing food and feed, and inhibition of mycotoxin absorption into the digestive tract. Nevertheless, if mycotoxin contamination already exists, application of decontamination methods is advised (Jouany, 2007). Various methods for mycotoxin reduction were studied, including the application of irradiation, thermal or microwave heating, ozone, chemical compounds, and microbials (Binder and Binder, 2004; Bullerman and Bianchini, 2007; Herzallah et al., 2008; Garg et al., 2013; Krstović et al., 2021). Moreover, the use of existing feed additives, such as enzymes showed a potential for degradation of some mycotoxins (Jakšić et al., 2022). Finally, measures can be applied for inhibition of mycotoxin absorption in the gastrointestinal tract, e.g., the use of adsorbents to reduce the bioavailability of mycotoxins in the digestive tract of animals (Abdel-Wahhab and Kholif, 2010). The use of adsorbents is one of the most suitable options for treatment of mycotoxins in practice (Li et al., 2018). In general, the adsorption method includes both physical and chemical force,

which can not only reduce the toxic impact of mycotoxins, but also enable the avoidance of toxic residues, thus becoming the most commonly applied method of protecting animals from mycotoxins (Li et al., 2018). Adsorbents can be used as single formulations, but are recently combined for better efficiency, usually as mineral-organic adsorbent mixture (Nešić et al., 2020).

The aim of this study was to examine the possibility of using two types of mycotoxin adsorbents. The first is pyrophyllite, a non-metallic aluminosilicate mineral that has adsorptive properties, primarily for ions of heavy metals and colors from aqueous solutions. The second adsorbent included two preparations of ground peach stones of different particle sizes. This type of adsorbent is organic, its waste biomass of agro-industrial source, and as an economically and ecologically profitable material, it is generally considered cheaper than mineral (inorganic) adsorbents. The adsorptive capacity of these preparations was tested for the adsorption of DON and OTA under *in vitro* conditions.

MATERIAL AND METHODS

Adsorbents

Adsorption potential test was performed for two preparations. Pyrophyllite, which belongs to the group of non-metallic aluminosilicate minerals, was selected as a potential inorganic adsorbent. Its use is already permitted in animal feed at up to 2% to prevent ruminal acidosis in ruminants (US Food and Drug Administration, 2019; Adamović et al., 2020). Another potential adsorbent is a preparation of ground peach stones (Lopičić et al., 2013). It was prepared in two fractions: larger (particle size d = 0.1 mm) and smaller (particle size d < 0.1 mm) fraction. The preparations were not commercially available. All preparations were prepared by and obtained from the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade, Serbia.

Mycotoxin removal procedure in vitro

To simulate *in vivo* conditions, where mycotoxin adsorption is performed on adsorbents, mycotoxin adsorption experiments were performed *in vitro* in solutions at pH 3 and 7. These pH values are expected in the digestive tract of monogastric animals and humans. The crude extract of DON, produced in house on wet maize kernels after artificial inoculation with toxigenic *Fusarium graminearum* (Krstović et al., 2018), as well as the standard solution of OTA (Sigma, St. Louis, MO, USA) were used for the adsorption experiments. 90 mg

of adsorbent was weighed into glass tubes using Teflon stoppers (volume of 20 cm^3), and then 10 mL of 0.1 M KH2PO4 (13.609 g KH2PO4 in 1000 mL of water, pH set to pH = 3 and pH = 7) was added. After that, a volume of 100 µL of crude toxin solution (c = 300 µg/mL) was added in order to obtain the final concentration of 3 µg/ml (crude toxin to adsorbent ratio was 1:3000). Mixing magnets were added to the tubes and then placed in an incubation system at 37 °C for 120 minutes with continuous mixing. The control was set up in the same way, without adsorbent. After the contact of the adsorbent and the mycotoxin was completed, the solution was filtered through quantitative filter paper for slow filtration and then additionally through nylon micro syringe filters with a porosity of 0.2 µm. The solutions prepared in this way were analyzed for HPLC for the mycotoxins' contents. All experiments were performed in duplicate. The adsorption efficiency of the natural adsorbent is expressed as an adsorption index, where:

Ci – mycotoxin initial concentration,

Ceq – mycotoxin concentration at equilibrium.

Adsorption index, % = [((Ci – Ceq)/Ci)] × 100

HPLC analysis

DON and OTA determinations were carried out on a 1260 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) using photodiode (DAD) and fluorescence (FLD) detectors (Agilent Technologies, USA) and a Hypersil ODS (150 x 4.6 mm i.e., particle size 5 µm) column (Agilent Technologies, USA). Stock calibration solutions at a concentration of 0.1 mg/mL were prepared in a mixture of ethyl acetate and methanol (19:1, v/v) for DON (Sigma, St. Louis, MO, USA), and in acetonitrile for OTA (Sigma, St. Louis, MO, USA). Working calibrant solutions were prepared by measuring the appropriate volume of the stock solution, evaporating to dryness under a stream of nitrogen at 50 °C and dissolving in the appropriate volume of the mobile phase. The stock solutions were stored at a temperature of -18 °C, while the working solutions were stored in a refrigerator at a temperature of 4 – 8 °C. HPLC conditions for DON determination were set as proposed by Abramović et al. (2005) and for OTA a method by Sugita-Konishi et al. (2006) was used. All analyses were done in duplicate. A volume of 20 µL of solution obtained after adsorption was injected into a HPLC system. The mobile phase consisted of an isocratic mixture of acetonitrile and water (HPLC grade, Sigma, St. Louis, MO, USA). For the determination of DON, the ratio of the solvents was the following: acetonitrile: water (16:84, *v/v*), and in the case of OTA, this ratio

acetonitrile: water was (50:50, *v/v*) and 1% of acetic acid was added (HPLC purity, Fisher Scientific, USA). In both cases, a mobile phase flow rate of 0.8 mL/min was applied. Before use, the mobile phase was filtered through regenerated cellulose membrane filters (0.45 μm) (Agilent Technologies, USA). DON was detected using a DAD detector at a wavelength of λ = 220 nm, for the detection of OTA, FLD detector was used at wavelengths of λ_{ex} = 333 nm and λ_{em} = 470 nm. The identification of DON and OTA was performed by comparing the retention times and spectra of the standards with the retention times and spectra of the samples.

RESULTS

The results of mycotoxin adsorption using the *in vitro* method are shown in tables 1-3.

Table 1. Results of mycotoxin adsorption using pyrophyllite, n = 2

		DON	**OTA**
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.53	0.17
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2945 ± 23	2569 ± 21
	A, %	0.03	13.47
pH 7	Ci, ng/mL	2978 ± 31	2980 ± 11
	Ceq, ng/mL	2974 ± 13	2948 ± 32
	A, %	0.15	1.1

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

Table 2. Results of mycotoxin adsorption using the preparation of ground peach stones (GPS), particle size d = 0.1 mm, n = 2

		DON	OTA
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.5	0.2
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2940 ± 56	1948 ± 89
	A, %	0.20	34.41
pH 7	Ci, ng/ml	2978 ± 31	2980 ± 11
	Ceq, ng/ml	2425 ± 38	2972 ± 12
	A, %	18.57	0.29

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

Table 3. Results of mycotoxin adsorption using the preparation of ground peach stones (GPS), particle size d < 0.1 mm, n = 2

		DON	OTA
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.5	0.2
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2941 ± 28	1701 ± 98
	A, %	0.19	42.71
pH 7	Ci, ng/mL	2978 ± 31	2980 ± 11
	Ceq, ng/mL	2379 ± 89	2897 ± 56
	A, %	20.11	2.80

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

The adsorption index of DON was below 1% using pyrophyllite for both pH values, as well as in the case of using the preparation of ground peach stones at pH = 3 (Figure 1). As for the preparation of ground peach stones

at pH = 7, adsorption index values of 18.57% (larger fraction) and 20.11% (smaller fraction) were obtained. This indicates that there is a certain potential of the ground peach stone preparation for DON adsorption at pH = 7, while the adsorption potential of pyrophyllite has not been proven.

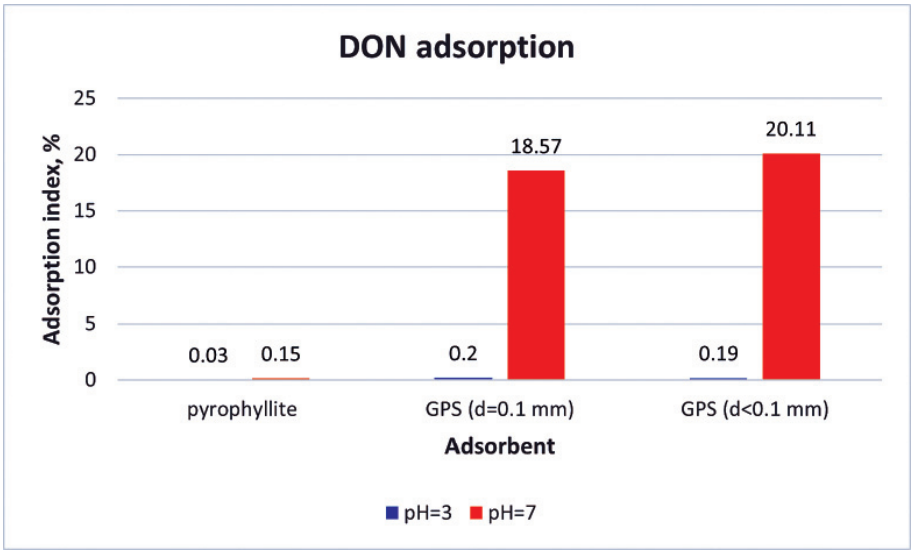

Figure 1. Comparative overview of the adsorption index of ground peach stones (GPS) and pyrophyllite for DON adsorption

When it comes to the adsorption index of pyrophyllite for OTA, it was affected by the pH value (Figure 2). Namely, at pH=3, the adsorption index was found to be 13.47%, while at pH = 7, it was as low as 1.1%. However, using the preparation of ground peach stones, these differences in the adsorption index were opposing compared to the application of pyrophyllite, where high values of the adsorption index of 34.41% (larger fraction) and 42.71% (smaller fraction) were found at pH = 3, and low values (0.29% and 2.80%) at pH = 7. These results indicate that the potential of the preparation of ground peach stones for OTA adsorption is higher compared to the potential of pyrophyllite, but there are still differences in their efficiency depending on the pH value of the environment in which the adsorption takes place.

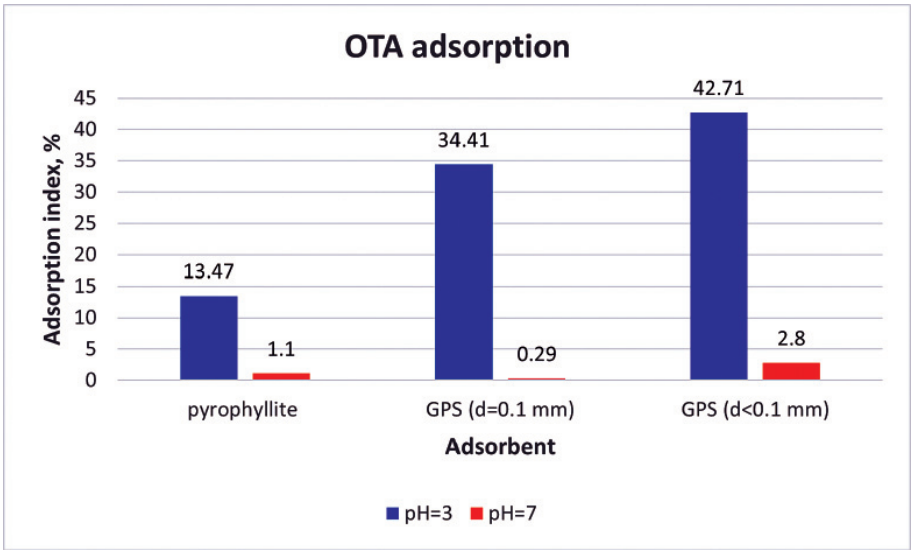

Figure 2. Comparative overview of the adsorption index of ground peach stones (GPS) and pyrophyllite for OTA adsorption

DISCUSSION

Pyrophyllite is already a recognized adsorbent of various substances such as cationic (due to negative hydrophilic surface sites) and anionic (after the addition of trivalent aluminium cation) dyes (Gücek et al., 2005). It is also known for its ability to bind ions of heavy metals such as cobalt (98.97%), lead (97.53%), nickel (98.87%) and cadmium (98.65%) from aqueous solution (maximum adsorption in optimized conditions) (Panda et al., 2018). Furthermore, in the research conducted by Murtić et al. (2020), it was demonstrated that the application of pyrophyllite, due to its adsorbent and ion exchange capabilities can reduce the use of mineral fertilizers in the production of lettuce without adverse effects on its yield and quality, in the amount of 25% or 50% of the recommended amount of fertilizer. However, despite the results of the aforementioned research, as well as the proven adsorption capacity of numerous other aluminosilicates against mycotoxins, literature data on the adsorption of mycotoxins, specifically by pyrophyllite, is very scarce. In contrast, the potential of peach stones to adsorb mycotoxins has been studied more extensively. Hence, in the research by Adamović et al. (2013) the adsorption of the following six mycotoxins was tested: AFB1, zearalenone, diacetoxyscirpenol (DAS), T-2 toxin, OTA and DON using the *in vitro* method. Similar to our study, the adsorption index was tested at two pH values, pH = 3.0 and 6.9.

Peach stones showed the highest affinity to aflatoxin (80.00% and 73.30%) and the lowest to DAS (0% at both pH values). When it comes to OTA and DON, a higher adsorption capacity was demonstrated in comparison with our research, given that the adsorption index at pH=3.0 and 6.9 were 66.67% and 50% for OTA, and 25% and 50% for DON, respectively. However, there were certain differences in the experimental design that could produce a different result (the ratio of individual mycotoxins and adsorbents in this research was 1:5000 and the particle size of the investigated biomass < 100 μm). Comparable research was conducted by Lopičić et al. (2013), at pH values of 3.0 and 7.0. Similar to the previous study, the best adsorption index of peach stones was obtained for aflatoxin (58.82% at both pH values), the poorest for DAS (0% and 16.67%), while for OTA it was 66.67% and 19.98% and 23.08% and 39.97% for DON, at pH 3.0 and 7.0, respectively. It is interesting to point out that in both studies, in addition to the unmodified, modified peach stones (activated with 1 M HCl) were used, and they achieved a somewhat different, weaker result compared to the unmodified. Furthermore, in the research by Bočarov-Stančić et al. (2011), the adsorption capacities of mineral adsorbents bentonite, diatomite and zeolite against aflatoxin, DAS, OTA, and DON were investigated. Like in the previous research, the highest adsorption index was obtained for aflatoxins (at pH 3.0 and 6.9: bentonite 96.90%; zeolite 95.50% and diatomite 95%) which is slightly higher compared to organic adsorbents, while the weakest was obtained for DAS (0% in all adsorbents). However, it is noted that organic adsorbents were more effective for OTA and DON. With OTA, only diatomite had adsorption properties (66.67%) at pH = 3. On the other hand, at pH = 6.9, as well as for bentonite and zeolite at both pH values, the adsorption index was 0%. Considering DON, mineral adsorbents showed affinity at pH = 3, bentonite and zeolite 50% and diatomite 25%, whereas at pH = 7 they did not display any affinity (0%). These results may lead to a conclusion that mineral adsorbents are more effective when it comes to aflatoxins, while organic adsorbents are more successful for OTA and DON adsorption. This was confirmed by the results of our research, where pyrophyllite was used as a mineral adsorbent. This adsorbent had an effect (13.47%) only for OTA at pH = 3, while it was ineffective in other cases. Ground peach stones were more efficient, as a larger diameter showed the degree of adsorption for OTA at pH = 3 (34.41%) and DON at pH 7 (18.57%), and the smaller diameter similarly, for OTA at pH = 3 (42.71%) and for DON at pH = 7 (20.11%). However, it is certainly necessary to prove this result under *in vivo* conditions.

The obtained results in our and similar research indicate that the degree of binding depends primarily on the type of adsorbent (mineral or organic), and

on individual types within these groups, as well as on the mycotoxin. These characteristics should be considered when creating formulations for use in practice, and certain adsorbents should be used depending on the conditions on the farm and occurrence of certain types of mycotoxins. In the future, formulations containing several types of adsorbents need to be tested, in order to enable action against several types of mycotoxins or use one of them that has the widest spectrum of adsorption capacity when different types of mycotoxins are involved.

CONCLUSION

The results of this study point to several conclusions. Namely, the potential of the preparation of ground peach stones for the adsorption of DON at pH = 7 was found, while its application at pH=3, the adsorption potential was not confirmed. The adsorption potential of pyrophyllite was not established at any of the pH values to which it was applied. The potential of the preparation of ground peach stones for OTA adsorption is higher compared to the potential of pyrophyllite, but there are differences in their efficiency depending on the pH value of the environment in which the adsorption takes place. Like with other *in vitro* research, this research cannot fully simulate conditions in the gastrointestinal tract of animals, so further *in vivo* experiments are necessary in order to evaluate the effectiveness of these materials as mycotoxin adsorbents.

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Author's Contribution:

S.K. basic idea, conception and design; D.G. acquisition of data; J.K. interpretation of results, manuscript revision; M.D. acquisition of data, drafting the manuscript; I.J. conception and design, final approval.

Competing interest

The author(s) declare that they have no competing interests.

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ISOLATION, MOLECULAR IDENTIFICATION AND ANTIBIOTIC SENSITIVITY OF *SALMONELLA* FROM BUFFALO FECES IN SYLHET DISTRICT OF BANGLADESH

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Abstract

Salmonella is a widely distributed foodborne pathogen affecting humans and animals around the globe. This cross-sectional bacteriological study was aimed at isolation of *Salmonella* from fecal samples of buffalos in Sylhet district (Upazilas Jaintapur and Fenchuganj) of Bangladesh, their molecular confirmation, and learning their antibiotic sensitivity patterns. A total of 334 fecal samples were collected using a simple random sampling technique. Standard conventional bacteriological culture and biochemical tests were done to isolate and confirm *Salmonella*. The isolates were confirmed with polymerase chain reaction (PCR). In this study, *Salmonella* was isolated from 56 samples (16.77%) using bacteriological culture methods and biochemical tests, and all isolates were confirmed in PCR tests. The prevalence of *Salmonella* was estimated to be 17.57% and 13.93% in Jaintapur and Fenchuganj Upazilas, respectively. Buffalo calves under one year of age had a higher prevalence (24.32%) than older animals aged 1 - 2 (18.62%), 2 - 4 (13.25%), and > 4 years (10.67%). Furthermore, the prevalence was substantially higher in diarrheic animals (72.22%) than in their healthy counterparts (13.60%). It did not vary significantly ($p > 0.05$) between animals from Jaintapur and Fenchuganj Upazilas. Likewise, no significant difference ($p > 0.05$) in *Salmonella* isolates was detected between different age groups. The results revealed that the isolation rate of *Salmonella* was significantly ($p < 0.05$) higher in female and diarrheic animals.

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The most effective antibiotics against the majority of *Salmonella* isolates were gentamycin (100%), levofloxacin (100%), and ciprofloxacin (76.67%). On the other hand, *Salmonella* isolates were highly resistant to tetracycline (100%), ampicillin (87.5%), and streptomycin (78.6%). Pathogenic microorganisms in feces are a potential risk to public health due to environmental and animal food contamination. Therefore, infection control and establishing strategic antibiotic therapy should be a priority.

Key words: Bangladesh, buffalo feces, *Salmonella*, *inv*A gene, antibiotics sensitivity

IZOLACIJA, MOLEKULARNA IDENTIFIKACIJA I ANTIBIOTSKA OSETLJIVOST *SALMONELLA* IZ BIVOLJEG IZMETA U OKRUGU SILHET, BANGLADEŠ

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Kratak sadržaj

Salmonella je široko rasprostranjen patogen koji se prenosi hranom i pogađa ljude i životinje širom sveta. Ova bakteriološka studija poprečnog preseka imala je za cilj izolaciju *Salmonella* iz uzoraka fecesa bivola u okrugu Silhet (distrikti Jaintapur i Fenchuganj) u Bangladešu, njihovu molekularnu potvrdu i osetljivost na antibiotike. Ukupno 334 uzorka fecesa prikupljena su jednostavnom tehnikom slučajnog uzorkovanja. Standardne konvencionalne bakteriološke metode i biohemijski testovi su urađeni da bi se izolovala i potvrdila *Salmonella*. Izolati su potvrđeni lančanom reakcijom polimeraze (PCR). U ovoj studiji *Salmonella* je izolovana iz 56 uzoraka (16,77%) metodom kultivacije i biohemijskim testovima, a svi izolati su potvrđeni i PCR testovima. Prevalencija *Salmonella* je iznosila 17,57% i 13,93% u Jaintapuru i Fenchuganj distriktu. Mladunčad bivola mlađih od jedne godine imala

su veću prevalenciju (24,32%) od starijih životinja uzrasta 1 - 2 (18,62%), 2 - 4 godine (13,25%) i > 4 godine (10,67%). Osim toga, prevalencija je bila znatno veća kod životinja sa dijarejom (72,22%) nego kod zdravih životinja (13,60%). Prevalencija nije značajno varirala ($p > 0,05$) između životinja iz Jaintapura i Fenchuganj distrikta. Isto tako, nije otkrivena značajna razlika ($p > 0,05$) u izolatima *Salmonella* između različitih starosnih grupa. Rezultati su otkrili da je stopa izolacije *Salmonella* značajno ($p < 0,05$) veća kod ženki i životinja sa dijarejom. Najefikasniji antibiotici protiv većine izolata *Salmonella* bili su gentamicin (100%), levofloksacin (100%) i ciprofloksacin (76,67%). S druge strane, izolati *Salmonella* su bili visoko otporni na tetraciklin (100%), ampicilin (87,5%) i streptomicin (78,6%). Patogeni mikroorganizmi u fecesu predstavljaju potencijalni rizik za javno zdravlje zbog kontaminacije životne sredine i hrane za životinje. Stoga, kontrola infekcije i uspostavljanje strateške antibiotske terapije treba da budu prioritet.

Ključne reči: Bangladeš, bivolji feces, *Salmonella*, *inv*A gen, antibiotska osetljivost

INTRODUCTION

Salmonella, a genus of the family *Enterobacteriaceae*, is a major cause of foodborne disease of public health significance both in developed and developing countries (Gebeyehu et al., 2022). All across the world, pathogens have been isolated from the feces of animals (Sabur et al., 2021). Salmonellosis, the clinical form of *Salmonella* infection, is a costly disease to livestock producers on account of mortality, treatment expenses, reduced milk yield, and weight loss/decreased weight gain within the herd (Callaway et al., 2005). By producing high-quality milk, meat, and farmyard waste, buffalo ranching contributes significantly to the agriculture and cattle sectors in many Asian nations (Khongsai, 2020). Bangladesh has only 1.485 million indigenous buffaloes, with a more significant concentration (40%) in coastal areas and certain buffalo pockets. Smallholder farmers in Bangladesh raise a small number of imported Nili-Ravi and Murrah buffaloes and their crosses on a few fields (Samad, 2020). Pathogens that infect buffaloes and their offspring or are transferred via their products are significant because they impact milk production and total livestock performance. In buffaloes, *Salmonella* is a major pathogen causing calf diarrhea (Khongsai, 2020; Ribeiro et al., 2000), leading to early-age

calf mortality (Rana et al., 2012) and a foremost pathogen transmitted through animal products. *Salmonella enterica* subsp. *enterica* can cause infections with a variety of clinical signs, as well as systemic infections characterized by diarrhea and septicemia, and, in extreme cases, death.

Antimicrobial resistance (AMR) is now recognized as one of the most serious global threats to human health in the 21st century (Morehead and Scarbrough, 2018; Tacconelli et al., 2018a; Tacconelli et al., 2018b) and a decline in the rate of new antibiotic development (Luepke and Mohr, 2017). It causes a considerable risk of death and economic hardship all over the world. However, impoverished nations are more harmed due to extensive antibiotic abuse, non-human antibiotic usage, poor medication quality, and insufficient surveillance (Ayukekbong et al., 2017; Van Boeckel et al., 2019). Bangladesh, a Southeast Asian developing country with a high level of AMR, poses a regional and global concern. Several studies have found irrational antibiotic prescribing by doctors and veterinarians, a patient habit of self-medication, and indiscriminate antibiotic usage in agriculture and farming in various sections of the nation (Biswas et al., 2014; Sutradhar, 2014).

Salmonella species with single and multidrug resistance are becoming more common (Liu et al., 2010). Salmonellosis is treated with a variety of antibiotics. Fluoroquinolones and third generation cephalosporins are the most often prescribed antibiotics to treat salmonellosis. Chloramphenicol, Ampicillin, Amoxicillin, and trimethoprim-sulfamethoxazole are some of the older antibiotics occasionally used as alternatives (Ayukekbong et al., 2017). Antimicrobial resistance in *Salmonella* has been observed to be on the rise in several developing and developed nations (Schmidt et al., 2018).

In some upazilas of the Sylhet region, the farmers depend upon the buffaloes primarily for draught and meat purposes, an integral part of the farming system in this area. Buffalo is economically significant in this region and could be considered a "small tractor" for farmers, and its meat and milk are nutritionally rich. Despite their great contribution to the socio-economy, they are still undervalued. Diseases are one of the most significant constraints for buffalo production, which decrease milk and meat production and reduce farmer's income. In addition, the status of *Salmonella* in buffalo populations in Sylhet district is unknown. Therefore, this study aimed to isolate and identify *Salmonella* from buffalo feces by conventional bacteriological, biochemical, and molecular methods and to determine the antibiotic sensitivity of *Salmonella* isolates.

MATERIAL AND METHODS

Study Area and Sample Collection

For this study, Fenchuganj and Jaintapur Upazilas of Sylhet District of Bangladesh were purposively selected due to their relativeness of larger buffalo population and milk production. The sampling units for the study were buffalo of different ages. A total of 334 fecal samples of buffaloes (112 from Fenchuganj and 222 from Jaintapur) of Sylhet District constituted the study population, which was selected by using a simple random sampling method. The study was conducted for a period of one year (July 2021 to June 2022). Feces samples were collected directly from the rectum and freshly voided feces from buffalo. The samples were placed in a labeled clean plastic container and immediately transferred to the laboratory for further analysis.

Isolation and identification of Salmonella

Conventional bacteriological methods were used to isolate the *Salmonella* from feces samples. The samples were inoculated into buffered peptone water (BPW), incubated at 37°C for 24 hours, and 0.5 mL was transferred to 10 mL Tetrathionate Broth (Merck) and incubated at 37°C for 24 hours. Following enrichment, a loopful of samples were streaked on xylose-lysine deoxycholate (XLD) agar and *Salmonella – Shigella* (SS) agar plates and incubated at 37 °C for 24 h. Biochemical confirmation of *Salmonella* was performed using TSI agar, Urea agar, Simmon's citrate agar, methyl red, and Voges Proskauer broth.

Bacterial genomic DNA Extraction and PCR assay

The DNA extraction was performed according to the manufacturer's instructions using the Addprep genomic DNA extraction kit (Addbio Inc. Ltd., Korea). These eluted DNA samples were stored at -80 °C until further analysis. The PCR analysis was performed to detect the *Salmonella* invasion gene (*invA* gene) according to the manufacturer's instructions (Addbio Inc. Ltd., Korea). PCR assay performed in the thermal cycler TC1000G PCR System® (DLAB Scientific Inc., USA) with a heated lid. The cycling conditions included 50 °C for 3 minutes (UDG Reaction), 95 °C for 10 minutes (Initial Denaturation), 35 cycles of 95 °C for 30 seconds (denaturation), 68 °C for 45 seconds (annealing), and 72 °C for 5 minutes for final extension (Khan et al., 2021). For the detection of *Salmonella*, the primers of invS-F (5'-TAA TGCCAGACGAAAGAGCGT-3') and invS-R (5'-GATATTGGTGTTTATGGG GTCGTT-3') were

used (Khan et al., 2021). All reaction mixtures, including the negative control and *Salmonella* positive DNA, were tested in duplicate in the same run of PCR assay. PCR products were analyzed on 1.8% agarose gels stained with RedSafe™ (iNtRON Biotechnology, Korea) Nucleic Acid Staining Solution (20,000×), photographed, and stored as a digital image.

Determination of Antibiotic sensitivity patterns

The *in vitro* antibiotic sensitivity test was determined by the standard disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI, 2020). The antibiotic discs used in the present study were Ampicillin (AMP, 10 µg), Amoxicillin/Clavulanic Acid (AMC, 30 µg), Gentamycin (CN, 10 µg), Streptomycin (S, 10 µg), Cefixime (CFM, 5 µg), Ceftriaxone (CRO, 30 µg), Azithromycin (AZM, 15 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Colistin sulfate (CT, 10 µg), Tetracycline (TE, 10 µg), Sulfamethoxazole-trimethoprim (SXT, 25 µg) all from Oxoid company, UK. After incubation, each disc's diameter of the clear zones produced by antibacterial inhibition of bacterial growth was measured to the nearest millimeter and then classified as resistant, intermediate, or susceptible using a published interpretive chart of Clinical and Laboratory Standards Institute (CLSI,2020).

Statistical analysis

Microsoft Excel Office 2010 was used for descriptive statistics. Chi-square tests were used to assess the significance of differences in prevalence between age, sex, and health status of the animals. P values less than 0.05 were considered significant using Chi-square tests (SPSS Inc., Chicago, IL, USA).

RESULTS

Salmonella was isolated from 56 (16.77%) out of 334 examined samples, of which 39 (17.57%) and 17 (13.93%) were from feces of Jaintapur and Fenchuganj upazila, respectively (Table 1). The prevalence of *Salmonella* was higher in buffalo calves aged less than one year (24.32%); although the difference was not statistically significant ($p = 0.11$) (Table 2). Furthermore, the isolation rate was relatively higher in animals aged 1 - 2 years (18.62%) than in the groups of 4 years and above four years (Table 2).

Table 1: Overall prevalence of *Salmonella* in buffalo feces and its association with study area

Study area	Number of samples tested	Tests name					
		Culture positive	Prevalence	Biochemical Test positive	Prevalence	PCR positive	Prevalence
Jaintapur	222	45	20.27%	39	17.57%	39	17.57%
Fenchuganj	122	19	15.57%	17	13.93%	17	13.93%
Total	334	64	19.16%	56	16.77%	56	16.77%

Table 2: Association of the prevalence of *Salmonella* with age of buffalo populations

Age of animals	No. of animals examined	No. of positive fecal samples	Prevalence	P-value
Up to 1 year	74	18	24.32%	0.11
1 – 2 years	102	19	18.62%	
2 - 4 years	83	11	13.25%	
> 4 years	75	8	10.67%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

It was observed that out of the total 334 feces samples collected from two different upazilas, the female buffaloes showed a higher prevalence (21.56%) of *Salmonella* in their fecal samples (Table 3). All suspected *Salmonella* colonies underwent specific biochemical tests such as indole formation, methyl red and Voges Proskauer reaction, citrate utilization, and triple sugar iron agar due to their cultural and morphological characteristics. Sixty-four of the suspected *Salmonella* colonies had 56 confirmed colonies by biochemical test results. Most of the *Salmonella* isolates showed fermentation of glucose, gas

production from glucose, H_2S formation, but none of the isolates showed either lactose or sucrose. The results showed that the prevalence rate of *Salmonella* was statistically significant (p = 0.01). In this study, the *Salmonella* isolation rate was categorized based on the health condition of buffalo populations. The isolation rate of *Salmonella* was higher in diarrheic buffalo fecal samples (72.22%) compared with apparently healthy animals (13.60%). The result revealed statistically significant differences (p = 0.01) (Table 4).

Table 3: Association of *Salmonella* isolation rate with the sex of animals

Sex of animals	No. of animals examined	No. of positive fecal samples	Prevalence	*p*-value
Male	153	17	11.11%	0.01
Female	181	39	21.55%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

Table 4: The association of *Salmonella* isolation rate with the health status of buffalo populations

Health status of animals	No. of animals examined	No. of positive fecal samples	Prevalence	*p*-value
Apparently healthy	316	43	13.60%	< 0.01
Diarrheic	18	13	72.22%	
Total	334	56	16.77%	

Level of significance $p < 0.05$

A total of 56 isolates of *Salmonella* were tested for commonly used 12 antibiotics from 8 groups. All the *Salmonella* isolates were found resistant to one or more antibiotics used. This antibiotic susceptibility study revealed that *Salmonella* isolates were 100%, 93.33%, and 76.67% sensitive to Gentamycin, Levofloxacin, Colistin sulfate, and Ciprofloxacin. On the other hand, isolates were 100%, 87.5%, and 78.6% resistant to Tetracycline, Ampicillin, and Streptomycin, respectively (Table 5.). All of the biochemically positive *Salmonella* isolates

(n = 56) were submitted to a PCR test using a particular primer set to identify the *invA* gene of *Salmonella*. After agarose gel electrophoresis under UV transilluminator, all the biochemically identified 56 isolates were confirmed positive for *Salmonella* and revealed *an invA* gene-specific band at 100bp (Figure 1.).

Table 5. Antibiotic susceptibility profile of *Salmonella* isolated (n = 56) from buffalo feces

Antibiotic tested	**Susceptible (%)**	**Intermediate (%)**	**Resistance (%)**
Penicillin			
Ampicillin (AMP, 10 µg)	-	7 (12.5)	49 (87.5)
Amoxicillin/clavulanic Acid (AMC, 30 µg)	30 (53.33)	15 (26.67)	11 (20)
Aminoglycosides			
Gentamicin (CN, 10 µg)	56 (100)	-	-
Streptomycin (S, 10 µg)	-	12 (21.4)	44 (78.6)
Cephalosporins			
Cefixime (CFM, 5 µg)	41 (73.21)	4 (7.14)	11 (20)
Ceftriaxone (CRO, 30 µg)	39 (70)	4 (7.14)	13 (23.21)
Macrolides			
Azithromycin (AZM, 15 µg)	39 (70)	-	17 (30)
Fluoroquinolones			
Ciprofloxacin (CIP, 5 µg)	43 (76.67)	13 (23.21)	-
Levofloxacin (LEV, 5 µg	56 (100)	-	-
Polymyxins			
Colistin Sulphate (CT, 10 µg)	52 (92.86)	-	4 (7.14)
Tetracyclines			
Tetracycline (TE, 10 µg)	-	-	56 (100)
Folate Pathway Inhibitors			
Sulfamethoxazole -Trimethoprim (SXT, 25 µg)	35 (62.5)	-	21 (37.5)

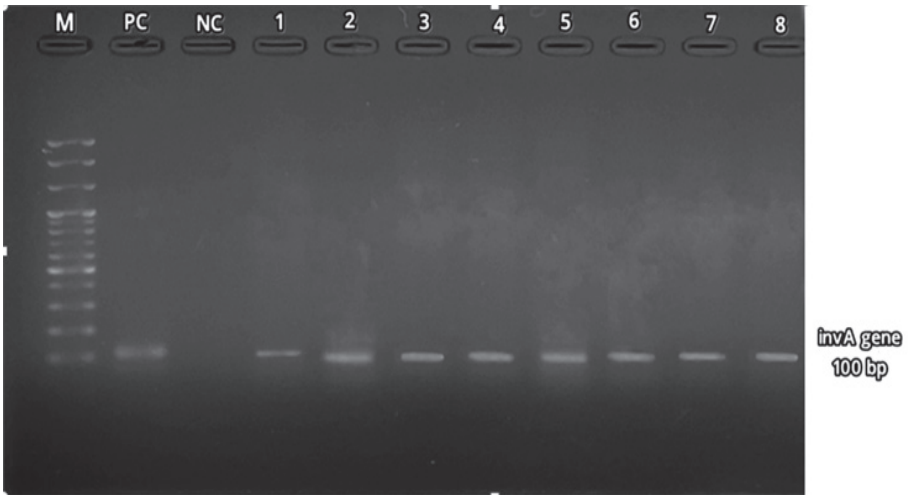

Figure 1. PCR amplification of biochemically identified *Salmonella*

DISCUSSION

In the present study, 334 buffalo fecal samples were collected from Jaintapur (222) and Fenchuganj (112) Upazilas of Sylhet district. Bacteriological examination revealed the *Salmonella* organisms from 56 of 334 buffalo fecal samples with a prevalence of 16.77% (Table 1), which is close to 11.2% (Zahran and Elbehiry, 2014). *Salmonella* in the buffalo population is a health risk for people who consume the products (meat, milk), as it has been reported that 25% of meat samples are contaminated with *Salmonella* (Abd-Elghany et al., 2022). The prevalence of *Salmonella* was higher in young (up to 1 year) and sick (diarrheic) animals, estimated to be 24.32% and 72.22%, respectively. The age-related result of the present study is consistent with the findings of other researchers (Saha et al., 2013; Mahmood et al., 2014). The findings also coincide with the results of Hunduma et al. (2010), who stated that diarrhea is a major problem in animals with a higher prevalence.

For detection of *Salmonella* genus using PCR, it was found that all PCR products, including positive control, resulted in 100 bp amplified fragment. Figure 1 revealed no amplified DNA fragments obtained from the non-*Salmonella* genus. *Salmonella* isolates produced a white band at the level of marker DNA (Figure 1). The PCR method is recommended for detecting *Salmonella* in feces samples because it is faster than culture methods (Stone et al., 1994; van der Zee and Huis in´t Veld, 2000).

According to Vella and Cuschieri (1995), *Salmonella* isolates were most frequently resistant to trimethoprim. *Salmonella* isolated from feces of dairy cattle were resistant to ampicillin (100%), streptomycin (66.7%), and tetracycline (55.56%) but susceptible to gentamicin (100%) and ciprofloxacin (91.7%) as reported by others (Khan et al., 2021). According to Wieczorek and Osek (2013), sulfamethizole resistance was found in various strains of *S.* Dublin and *S.* Enteritidis. *Salmonella* isolates are resistant to multiple antibiotics (ampicillin, streptomycin, tetracycline, and sulfamethizole). The isolates were susceptible to gentamycin (100%), levofloxacin (100%), and ciprofloxacin (76.67%) but have shown higher resistance to tetracycline, ampicillin, and streptomycin (100%, 87.5%, and 78.6%, respectively). According to Esaki et al. (2004), many *Salmonella* isolates from both healthy and ill animals were resistant to two or more antimicrobials. These microorganisms are disseminated in the environment and spread through the excrement of animals that appear to be in good health. According to reports, *S.* Typhimurium was released in a pig farm (Tanaka et al., 2014). The release was relatively high for several days following vaccination; therefore, the animals appeared to be in good health. As a result, herd infection could come even from the excrement of clinically healthy animals. Furthermore, a range of serotypes of antibiotic-resistant bacteria may exist. *Salmonella* isolates were shown to be resistant to numerous drugs in the current investigation. Tetracycline resistance was found in all *Salmonella* isolates. The results of this study revealed that *Salmonella*-infected buffalo could be effectively treated with gentamycin, Levofloxacin, and Ciprofloxacin.

CONCLUSION

It could be concluded that *Salmonella* is a significant cause of diarrhea in buffaloes with salmonellosis in Bangladesh. Therefore, a rapid and proper diagnosis could prevent harm inflicted on the livestock industry. The molecular basis of *Salmonella* identification techniques, such as using the *invA* gene-specific PCR method, could be helpful in diagnostic and research laboratories. This study suggests the strategic use of antibiotics to control *Salmonella* infections in animals.

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Author's Contribution:

MMH and MMR: Supervised and contributed to study design; BDG and RB: Carried out the laboratory experiments; SA and MSRC: Performed statistical analysis; and BDG and MMH: Wrote the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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EVALUATION OF PHYSIOLOGICAL BIOMARKERS AS POSSIBLE PREDICTIVE FACTORS AND PROGNOSIS MARKERS OF KIDNEY INJURY IN DOGS NATURALLY INFECTED WITH *LEISHMANIA INFANTUM*

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Abstract

Impaired renal function is one of the main characteristics of dogs infected with *Leishmania infantum*. Early diagnosis of kidney injury is essential for improving patient's prognosis. This study aims to evaluate physiological biomarkers as predictors of kidney injury and prognostic markers. Medical records of fifty-nine dogs of different breed, age, and sex, naturally infected with *L. infantum,* were analyzed. Red blood cells, leucocytes, platelets count, hematocrit, total plasma proteins, plasma globulin, plasma albumin, serum creatinine, serum urea, serum phosphorus, serum symmetrical dimethyl arginine, urine analysis, urinary density, urinary protein creatinine ratio, urinary creatinine, urinary protein, and systemic blood pressure were examined in trial 0. Six months after trial 0, twenty-four dogs returned for clinical and laboratory examination. The second medical record analysis was identified as trial 1. The twenty-four dogs were examined using the same tests performed in trial 0. The physiological biomarker such as platelets and leukocyte count, hematocrit, serum phosphorus, urinary density, and systemic blood pressure, showed a significant correlation as prognostic and predictive factors of kidney injury in dogs. The platelet count was used as the physiological biomarker to show the value as a predictive factor and prognostic marker related to biomarkers of kidney injury in dogs naturally infected with *L. infantum*.

Key words: acute kidney injury, CanL, survival

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EVALUACIJA FIZIOLOŠKIH BIOMARKERA KAO MOGUĆIH PREDIKTIVNIH FAKTORA I PROGNOSTIČKIH MARKERA OŠTEĆENJA BUBREGA KOD PASA PRIRODNO INFICIRANIH SA LEISHMANIA INFANTUM

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Kratak sadržaj

Oštećena bubrežna funkcija je jedna od glavnih karakteristika pasa zaraženih sa Leishmania infantum. Rana dijagnostika oštećenja bubrega je od suštinskog značaja za bolju prognozu stanja pacijenta. Ova studija ima za cilj da proceni fiziološke biomarkere kao prediktore oštećenja bubrega i prognostičke markere. Izvršena je analiza medicinske dokumentacije za pedeset i devet pasa različitih rasa, starosti i pola, prirodno zaraženih sa L. infantum. Crvena krvna zrnca, leukociti, broj trombocita, hematokrit, ukupni proteini plazme, globulini u plazmi, albumin u plazmi, serumski kreatinin, serumska urea, serumski fosfor, serumski simetrični dimetil arginin, analiza urina, gustina urina, odnos urinarnog proteina i kreatinina, kreatinin u urinu, urinarni protein, i sistemski krvni pritisak ispitivani su kao parametri nultog ispitivanja. Šest meseci nakon nultog ispitivanja, dvadeset i četiri psa su se vratila na klinički i laboratorijski pregled. Podaci od drugog pregleda su označeni kao ispitivanje 1. Dvadeset četiri psa su pregledana korišćenjem istih testova izvršenih u ogledu „0“ (nultog ispitivanja). Fiziološki biomarkeri kao što su broj trombocita i leukocita, hematokrit, fosfor u serumu, gustina urina i sistemski krvni pritisak, pokazali su značajnu korelaciju kao prognostički i prediktivni faktori oštećenja bubrega kod pasa. Broj trombocita je korišćen kao fiziološki biomarker da pokaže vrednost kao prediktivni faktor i prognostički marker koji se odnosi na biomarkere oštećenja bubrega kod pasa prirodno inficiranih L. infantum.

Ključne reči: akutno oštećenje bubrega, lajšmanioza pasa, preživljavanje

INTRODUCTION

Visceral leishmaniasis (VL) is an infectious, systemic, and zoonotic disease caused by the protozoan *Leishmania infantum* and is transmitted by infected female sandflies, Lutzo*myia longipalpis,* and its main vector is in Brazil (Dantas-Torres and Brandão-Filho, 2006). It is estimated that there are millions of infected dogs in South America, especially in Brazil, where there are high rates of infection (Marcondes and Day, 2019)

Infected dogs can develop canine leishmaniosis (CanL), which can affect several organs and therefore exhibit several clinical characteristics, ranging from apparently healthy to severe illness and death, depending on the immune response triggered by the patient (Soares et al., 2005; Freitas et al., 2012)

Renal function impairment is quite frequent among the clinical changes observed in infected animals (Braga et al., 2015). The formation and deposition of immune complexes secondary to CanL can cause glomerulonephritis and tubulointerstitial lesions (De Oliveira Frazilio et al., 2018). Some authors believe that tubulointerstitial lesions occur secondary to glomerulopathy (Pardo-Marín et al., 2017; De Oliveira Frazilio et al., 2018). Renal proteinuria, which is a reflection of increased glomerular capillary permeability, is associated with the production of immune complexes and may be evidence of kidney injury (D'Amico and Bazzi, 2003; Soares et al., 2005; Freitas et al., 2012; Brown et al., 2013; IRIS 2023). Treatments for CanL in Brazil are based on miltefosine for leishmanicidal action, immunomodulatory medications such as domperidone, and immunotherapy, which consists of three double doses of the Leishtec® vaccine with intervals of twenty-one days between applications. Subsequently, a six-monthly booster should be administered with the application of a double dose. Immunotherapy was a good and efficient protocol for reducing clinical manifestations and controlling CanL relapses compared to other treatments (Santos et al., 2007; Ribeiro et al., 2013; Araujo and Gondim, 2020).

This study aims to evaluate physiological biomarkers as predictive and prognostic markers of the evolution of infection, renal injury, and renal failure in dogs naturally infected with *L. infantum*, assisting in the determination of treatment procedures, managing possible alterations identified, and in the prognosis of patients.

MATERIAL AND METHODS

For the retrospective data collection, no ethical approval was required as no identifying information was included, and no dogs were actively recruited.

Medical records of fifty-nine dogs of different breeds and ages naturally infected with *L. infantum* were examined. Out of the fifty-nine dogs studied, twenty-six were female dogs, while thirty-three were males. The dogs were classified and divided into stages as suggested by Solano-Gallego et al. (2011). The clinical staging of canine visceral leishmaniasis is classified in four stages based on serological status, clinical symptoms and laboratory findings. Dogs in Stage I - Negative to low positive antibody levels; Dogs with mild clinical symptoms such as peripheral lymphadenomegaly, or papular dermatitis. Usually, no clinicopathological abnormalities were observed. Normal renal profile was the following: creatinine <1.4 mg/dl; non-proteinuric: UPC < 0.5. Stage II - Low to high positive antibody levels; Dogs, which apart from the signs listed in stage I, may exhibit the following symptoms: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis / onychogryphosis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), anorexia, weight loss, fever, and epistaxis; Clinicopathological abnormalities such as mild non-regenerative anemia, hyperglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome. Substages: a) Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5 b) Creatinine <1.4 mg/dl; UPC = 0.5-1,0. Stage III - Medium to high positive antibody levels; Dogs, which apart from the symptoms listed in stages I and II, may show the signs originating from immune-complex lesions: vasculitis, arthritis, uveitis, and glomerulonephritis. Clinicopathological abnormalities listed in IRIS stage II of chronic kidney disease (CKD), creatinine 1.4-2,8 mg/dl, or stage I with UPC > 1. Stage IV - Medium to high positive antibody levels; Dogs with clinical symptoms listed in stage III. Pulmonary thromboembolism, or nephrotic syndrome (marked proteinuria UPC > 5) and final stage renal disease; Clinicopathological abnormalities listed in IRIS stage II CKD, stage III (creatinine 2,9-5mg/dl) and stage IV (creatinine >5 mg/dl).

The dogs from stage II and those undergoing treatment with immunotherapy (do Nascimento et al, 2018; Gouveia et al, 2021) as suggested by Brasileish (2019) were included. Medical records should also present parasitological examinations for *Leishmania sp.* identification by direct or molecular identification or titration above 1:160 in the indirect Fluorescent Antibody Test (IFAT) or sample value four times higher than the cut-off point in the Enzyme-Linked Immunosorbent Assay (ELISA) (Brasileish, 2019; Solano-Gallego et al., 2011; Laia et al., 2011) at trial 0 (T0). Eighteen (30.5%) dogs were classified as stage IIa (Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5), fifteen (25.4%) as stage IIb (Creatinine <1.4 mg/dl; UPC = 0.5-1), twenty-three (39%) in stage III, and three (5.1%) dogs were classified as stage IV (Solano-Gallego, et al., 2011).

Also, medical records should present complete blood count tests: red blood cell count (RBC), hematocrit (HC), leucocytes (LE) and platelets (PL), total plasma proteins (PROT), plasma globulin (GL), plasma albumin (AL), serum creatinine (CR), serum urea (UR), serum phosphorus (PH), serum symmetrical dimethyl arginine (SDMA), urine analysis, urinary density (UR. D), urinary protein creatinine ratio (UPC), urinary creatinine (CR. U) and urinary protein (UR PTN), all performed in the exact consultation, in the same laboratory. Systemic blood pressure (SBP) was measured using vascular doppler or through the oscillometer method with the animal positioned in lateral decubitus, in line with criteria for cuff size and minimum stress. Three to seven measurements were performed, and the first measurement was discarded to obtain an average of the other measurements, considering the patient's SBP value.

Animals that showed signs of dehydration, episodes of vomiting, diarrhea, inappetence, or lower urinary tract disease were excluded from the study. This first moment is identified as T0. Six months after T0, twenty-four dogs were returned for clinical and laboratory evaluation. The second medical record analysis was identified as trial 1 (T1). These twenty-four dogs were clinically healthy and evaluated through the same exams performed in the same laboratory in T0. Thirty-five dogs were not returned for evaluation or returned for more than six months and were therefore excluded from the study.

Statistical Analysis

Principal component analysis (multivariate analysis) was performed using numerical variables. This analysis evaluated all numerical variables, considering their correlation, and was performed using Spearman's correlation matrix. Moderate, strong, and robust correlations were reported, with confidence intervals above 95%. Statistical analyses were performed using the R software version ("The R Project for Statistical Computing," 2019).

RESULTS

T0

The distribution of the dogs evaluated in this study is shown in Figure 1. The arrows facing the same direction indicate biomarkers that are positively correlated with each other. The points in the two quadrants on the right showed higher values for CR, UR, SDMA, PH, UPC, and UR. PTN. In these

quadrants there were higher concentration of dogs classified in stages III and IV, whereas those in the two left quadrants, mostly dogs classified in stages IIa and IIb, had higher values mainly for CR. U, HC, AL, and UR. D. The points in the two upper quadrants had higher PL counts. On the other hand, the points in the two lower quadrants had higher values, mainly for PROT and GL. There was a relative separation in the group IIa, with animals concentrated on the left side of the graph, and group IV, with animals focused on the right side. The chart shows the dispersion and evolution of dogs from T0 to T1. The dogs classified as stage IIa or IIb were in the left quadrant. The dogs in stages III and IV were in the right quadrant, with high levels of CR, UR, SDMA, PH, and UPC, demonstrating that patients classified in the final stages have a greater impairment of the glomerular filtration rate.

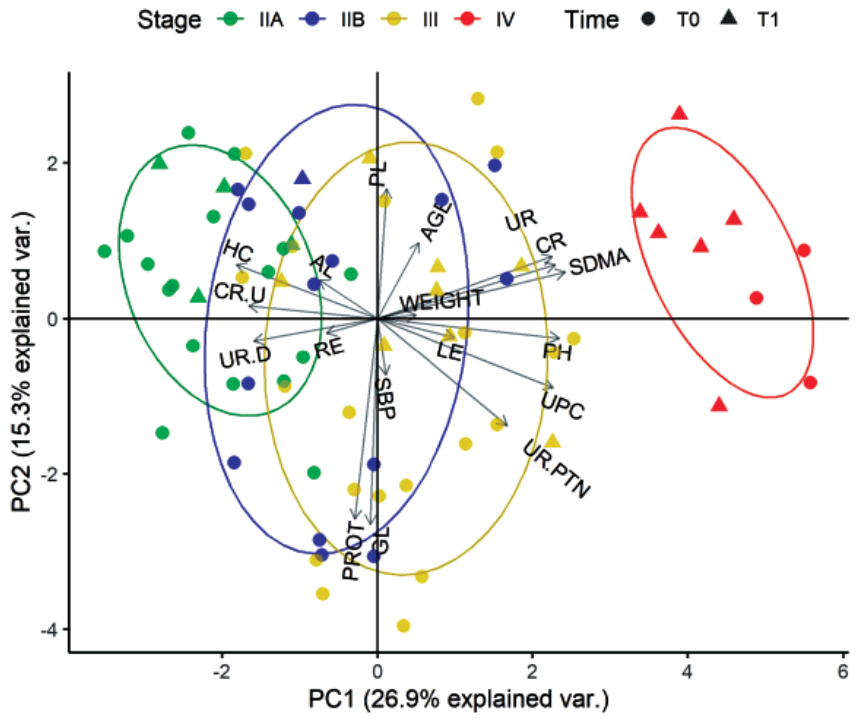

Figure 1: Dispersion of dogs considering the staging and the values obtained in the analytes studied at T0 and T1. The points in the two quadrants on the right showed higher values for CR, UR, SDMA, PH, UPC, and UR. PTN, whereas those in the two left quadrants showed higher values mainly for CR, UR, HC, AL, and UR. D. The points in the two upper quadrants had higher PL counts, while the points in the two lower quadrants had higher values, mainly for PROT and GL.

Anemia was found in twenty-one (35%) dogs out of fifty-nine analyzed in the study. Two (11%) of the dogs were classified as stage IIa, five (33%) as stage IIb, 11 (47%) in stage III, and three (100%) in stage IV present anemia.

Thrombocytopenia was present in seventeen (28%) dogs out of fifty-nine evaluated in the study. Three (16%) of the dogs classified as stage IIa, four (26%) in stage IIb, seven (30%) in stage III, and three (100%) in stage IV had thrombocytopenia.

Out of the thirty-three dogs at T0, classified by Solano-Gallego et al. (2011) IIa and IIb stages evaluated in this study, in thirteen (39%), UPC was the first biomarker to assess kidney injury to have the values higher than reference values. SDMA was the first evaluated biomarker with values higher than reference values in two dogs (6%). SDMA and UPC levels increased simultaneously in two dogs (6 %) classified by Solano-Gallego et al. (2011) IIa and IIb stages.

SDMA had the values above the reference (> 18 µg/dL IRIS, 2023) in one (5%) dog classified as stage IIa and in four (26%) dogs classified as stage IIb. In stage III, there was an increase in SDMA in eight (34%) dogs, and in stage IV in three (100%) dogs.

Analyzing correlations at T0, they were found to be moderate with values varying between 0.4 and 0.69, and strong between 0.70 and 0.89. SDMA had a moderate positive correlation with UR and CREAT and a moderate negative correlation with HC. UR levels had a strong positive correlation with serum CR levels and a moderate positive correlation with PH levels. PH showed a reasonable positive correlation with UPC and a moderate negative correlation with RBC and HC. PROT levels showed a moderate negative correlation with the PL count. AL levels had a moderate positive correlation with HC and RBC, which was negatively correlated with UPC and UR. PTN levels. UR. D showed a moderate correlation with CR.U.

Comparisons between T0 X T1

The correlation of biomarkers evaluated at T0 and T1 is shown in Table 1. When correlations were evaluated at T0 and T1, PL (T0) had a negative correlation with UPC and UR. PTN levels at T1. There was no correlation between PL and UPC in T0. The UR. D (T0) and UPC (T1) showed a strong negative correlation. LE (T0) had a moderate correlation with UR (T1). PH (T0) had a moderate correlation with UR. PTN.

In the analysis of the correlation between the variables in T0 and the variables of dogs with CR lower than 1.4 mg/L in T1 is shown in Table 2. UR D (T0) had a negative correlation with the UPC (T1) and the GL (T1). SDMA

(T0) was correlated with CR. U level (T1). PL (T0) correlated with UR and PH levels (T1). UR. PTN (T0) levels are associated with PROT levels (T1). PH and SBP (T0) were correlated with LE (T1). It was not possible to perform the analysis for animals with serum CR equal to or greater than 1.4 mg/L, as due to an insufficient number of dogs, it is not possible to ger a basis for interpretable analysis.

Table 1: Correlations between the biomarkers studied at T0 and T1 that were statistically significant.

BIOMARKER	TRIAL 0	BIOMARKER	TRIAL 1	CORRELATION	P
CR.U	T0	UR.D	T1	0.73	0.003
GL	T0	LE	T1	0.48	0.019
CR.U	T0	UR. PTN	T1	-0.71	0.023
PL	T0	UPC	T1	-0.69	0.023
PL	T0	UR. PTN	T1	-0.69	0.026
PROT	T0	GL	T1	0.45	0.027
UR. D	T0	UPC	T1	-0.69	0.027
SDMA	T0	CR. U	T1	0.68	0.029
CR. U	T0	UPC	T1	-0.68	0.03
LE	T0	UR	T1	-0.44	0.031
PROT	T0	LE	T1	0.43	0.031
PL	T0	LE	T1	-0.43	0.036
PH	T0	UR. PTN	T1	0.66	0.036
HC	T0	PTOT	T1	-0.42	0.043
UR. PTN	T0	UPC	T1	0.65	0.043
PL	T0	PH	T1	-0.41	0.048
SBP	T0	LE	T1	0.41	0.049

Hematocrit (HC), leucocytes (LE) and platelets (PL), total plasma proteins (PROT), serum globulin (GL), serum urea (UR), serum phosphorus (PH), serum symmetrical dimethyl arginine (SDMA), urine analysis, urinary density (UR. D), urinary protein creatinine ratio (UPC), urinary creatinine (CR. U), urinary protein (UR PTN) and systemic blood pressure (SBP).

Table 2: Correlation of the variables in T0 together with the variables of dogs that present creatinine lower than 1.4 mg/L at T1 that presented statistically significant.

BIOMARKER	TRIAL 0	BIOMARKER	TRIAL 1	CORRELATION	P
UR. D	T0	UPC	T1	-0.92	0.001
SDMA	T0	CR. U	T1	0.92	0.001
PL	T0	UR	T1	-0.59	0.013
UR. D	T0	GL	T1	0.57	0.016
SBP	T0	LE	T1	0.56	0.018
PL	T0	PH	T1	-0.55	0.022
PH	T0	LE	T1	0.53	0.03
UR. PTN	T0	PROT	T1	0.5	0.042

Hematocrit (HC), leucocytes (LE) and platelets (PL), total plasma proteins (PROT), serum globulin (GL), serum urea (UR), serum phosphorus (PH), serum symmetrical dimethyl arginine (SDMA), urine analysis, urinary density (UR. D), urinary protein creatinine ratio (UPC), urinary creatinine (CR. U) and urinary protein (UR PTN) and Systemic blood pressure (SBP).

DISCUSSION

The importance of evaluating coinfections in dogs infected with *L. infantum* is well known. Infections with *Babesia canis*, *Anaplasma platys*, *Hepatozoon canis* and *Ehrlichia canis* cause hematological alterations such as anemia, pancytopenia, and thrombocytopenia (Thrall, 2007; Vilela et al., 2013), which may change the values of laboratory tests in the studied dogs. The lack of this evaluation presents a limitation of this study.

Changes in the erythrogram of dogs infected with *L. infantum* were observed in this study, in the form of mild to moderate anemia and thrombocytopenia. However, the pathogenesis of anemia in CanL includes additional mechanisms such as reduced erythropoietin synthesis due to renal failure (Paltrinieri et al., 2016). Anemia may also result from the parasite's invasion of the bone marrow, which induces inflammation that may contribute to a decrease in erythrocyte production (Da Costa-Val et al., 2007). Anemia is usually present in normocytic and normochromic forms with a non-regenerative character (Freitas et al., 2012). Thrombocytopenia is a common finding in dogs with CanL and occurs due to vasculitis caused by circulating immune complexes,

thrombocytopoiesis disorders, and PL destruction (Solano-Gallego L., et al., 2009; De Carvalho Nicolato et al., 2013).

Hyperproteinemia is caused by hyperglobulinemia, which is associated with the activation of B lymphocytes and high antibody production (Freitas et al., 2012; Paltrinieri et al., 2016; Maia and Campino, 2018). A decrease in the production or renal loss of AL was also observed in this study, but the increase in GL levels was very expressive, causing the levels of PROT to increase significantly during CanL (Giunchetti et al., 2008; Solano-Gallego, et al., 2009). As for urinalysis, in the present study, proteinuria was the most frequent alteration in animals with CanL to both mild or severe degrees, as described by Amusategui et al. (2003) and Bonfanti and Zatelli (2004).

Glomerulonephritis, tubulointerstitial nephritis, and nephropathy are common in CanL (De Oliveira Frazilio et al., 2018). Initially, glomerulonephritis may manifest as asymptomatic proteinuria (Koutinas and Koutinas, 2014; Paltrinieri et al., 2016), but with its progression, excretion dysfunction can occur in the presence of an increase or decrease in the glomerular filtration rate (GFR) and systemic hypertension (Plevraki et al., 2006; Cortadellas et al., 2008). An increase in GFR associated with hypertension can amplify glomerulopathy, resulting in the progression of CKD (Koutinas and Koutinas, 2014). End-stage CKD is a severe manifestation of disease progression and the leading cause of death in CanL. The two main parameters used to classify the degree of kidney disease in CanL, according to (Solano-Gallego et al., 2011; Laia et al., 2011), are UPC, used as a marker of glomerular pathology, and serum CR level, used as a marker of renal excretion (Torrent et al., 2018).

In 2019, IRIS included SDMA levels in the CKD staging of chronic kidney disease. SDMA is methylated arginine produced by cellular catabolism correlated with CREAT and GFR (Nabity et al., 2015). Several studies have demonstrated that SDMA levels are elevated in dogs with CKD (Hall et al., 2014; Nabity et al., 2015). Studies have suggested that SDMA levels also increase earlier than serum CR levels (Hall et al., 2014; Nabity et al., 2015) because its production is not influenced by the loss of muscle mass. SDMA showed a moderate correlation with UR and PH in T0. An increase in SDMA, UR, PH, and CR and a reduction in UR is expected as there is a worsening of renal excretion and a decrease in TGF.

There is a paucity of data on the behavior of SDMA in dogs naturally infected with *Leishmania* sp., with only four studies reported to date (Pardo-Marín et al., 2017; De Oliveira Frazilio et al., 2018; Torrent et al., 2018; Giapitzoglou et al., 2020). The serum concentration of SDMA is elevated mainly in azotemic dogs with severe proteinuria and decreased UR. D (Giapitzoglou et

al., 2020). SDMA correlated with CR and UR. D, a result like that obtained by (Giapitzoglou et al., 2020). A study carried out by (Torrent et al., 2018) found different results, without 0observing a correlation between SDMA and CR.

SDMA was also inversely correlated with the red RBC and HC. Kidneys perform numerous metabolic functions, including their contribution to erythropoiesis. Several factors contribute to anemia in patients with reduced GFR and CanL. The reduction of erythropoietin of renal origin is an important cause, but other mechanisms, such as anemia of inflammatory origin, the influence of uremic toxins on the survival time of the erythroid lineage, cofactor deficiency, blood loss due to various bleeding events, and parasitic destruction (Babitt and Lin, 2012; Fiocchi et al., 2017; Lippi et al., 2021).

There was a significant and progressive increase in the number of dogs with SDMA levels above the reference values (IRIS, 2023) as the stage progressed. The expected result of disease progression between stages demonstrated that SDMA could be used to classify CanL severity (Giapitzoglou et al., 2020). A different result was found by Torrent et al. (2018), who found no significant difference in the comparison between the stages (Solano-Gallego et al., 2011).

In this study, two dogs classified in stage IIa (non-proteinuric and non-azotemic) with elevated SDMA levels were observed. (Cortadellas et al., 2008) pointed out that dogs with CanL and UPC levels between 0.2 and 0.5 may have a decrease in GFR. Dogs with CanL may have some renal perfusion impairment secondary to hypovolemia, which could cause an increase in SDMA concentration without an association with proteinuria or renal azotemia. Torrent et al. (2018) suggested that dogs with CanL and changes in SDMA concentration without proteinuria should be carefully examined for pre-renal causes of impaired renal perfusion.

UPC was shown to be an earlier diagnostic sign of acute kidney injury (AKI) concerning SDMA in dogs naturally infected with *L. infantum* observed in this study, which is in line with other studies (Pardo-Marín et al., 2017; Torrent et al., 2018; Giapitzoglou et al., 2020). Glomerulonephritis is the main pathological event of nephropathy in CanL, and proteinuria is the primary laboratory alteration. However, glomerular pathology can cause a reduction in GFR and an increase in the serum concentrations of SDMA and CR (Koutinas and Koutinas, 2014; Paltrinieri et al., 2016)

When evaluated as prognostic markers and predictors of kidney injury and kidney failure, the UPC and SDMA levels did not show a statistically significant correlation. Pardo-Marin et al. (2017) identified a reduction in the concentration of UPC, however, they did not observe a decrease in the SDMA levels in dogs after leishmanicidal treatment. In this study, we observed a cor-

relation between SDMA (T0) and CR. U (T1) levels; however, as the dogs examined in this study underwent a treatment with immunotherapy, an improvement in GFR may have occurred after the reduction of the injury caused by CanL. Gouveia et al, 2021 found that immunotherapy with vaccines was the most effective treatment for negative serology in the ELISA and IFAT tests after the treatments compared to other treatments in the study. The decrease in the result in the serology may suggest a reduction in the production of antigen-antibody complexes influencing the GFR.

An increase in PROT and GL levels, associated with a decrease in AL levels, has been observed in several studies (Artan et al., 2006; Sales et al., 2017). This increase in PROT and the presence of hyperglobulinemia are considered to be among the most common alterations in CanL and may be associated with increased levels of anti-*Leishmania* antibodies, especially in severe stages of the disease (Castro et al., 2012; Freitas et al., 2012). However, hypoalbuminemia observed in animals may be due to the migration of albumin to the extravascular environment, with the formation of edema, a widespread clinical change in CanL (Silva, 2007; Freitas et al., 2012) and/or associated with an inflammatory process and/or albuminuria (Pierantozzi et al., 2013; Proverbio et al., 2016). Given the processes that occur during CanL due to the association of GL with the stimulation of B lymphocytes (Vieira et al., 2021), we can explain why the serum concentration of GL and PROT (T0) had a significant correlation with LE (T1). AL levels did not significantly correlate with any of the biomarkers.

The development of glomerulopathies not only leads to complications resulting from the accumulation of uremic toxins and fluid and electrolyte imbalances, but also causes systemic arterial hypertension, aggravating the clinical picture of patients and possibly compromising other organs, such as the heart (Schiffrin et al., 2007). In this study, seventeen (28%) dogs were identified with SBP above 160 mmHg. Out of these, seven (41%) were non-azotemic and non-proteinuric. Cortadellas et al. (2008) observed that 49.5% (52 / 105) of the dogs had some degree of renal disease, and 61.5% (32 / 52) of these dogs were diagnosed with systemic hypertension (SH). Moreover, SH also was diagnosed in 2% of dogs without renal disease. Braga et al. (2015) found that all dogs with hypertension had histopathological and laboratory evidence of glomerular disease. However, there was no statistically significant correlation between elevated BP and the severity of glomerular lesions.

This study found that SBP (T0) correlated with LE count (T1). CanL glomerulonephritis can form large antigen-antibody complexes, inciting inflammation and overloading of glomerular capillaries, resulting in obstruction that can further elevate glomerular pressure (Harrison et al., 2012). There is also

evidence that inflammatory cells accumulate in the perivascular region of the kidney (Theuer et al., 2002) also contributing to hypertension.

Unlike SBP, data in the literature cite the destruction of LE in uremic patients (Minnaganti and Cunha, 2001; Cohen and Hörl, 2012; Pahl et al., 2010). In this study, predictive factors of LE (T0) and HC (T1) concerning UR were observed through an inverse correlation. Uremia is associated with hematological abnormalities such as hemostatic, granulocytic, lymphocytic, and PL disorders, mainly caused by chemotaxis, phagocytosis, and oxidative abnormalities. Some diseases related to antigen presentation have also been reported in uremic patients (Minnagantiand and Cunha, 2001; Cohen and Hörl, 2012) demonstrated that patients with renal failure had impaired body defense. Pahl et al. (2010) reported that the number of B lymphocytes and their ability to produce antibodies was reduced in patients with uremia. A study conducted on dogs infected with *Leishmania sp.*, showed more intense hematological alterations, such as profound anemia, thrombocytopenia, and leukopenia, characterizing pancytopenia associated with bone marrow hypoplasia or aplasia as a result of the invasion of the microorganism and immune-mediated processes (Paltrinieri et al., 2016). Hyperplasia and peripheral cytopenia can partially be attributed to the increased destruction of mature blood cells in the periphery. There are also morphologic features indicative of differentiation blockage and dyserythropoietic changes in the erythroid precursors (Poulaki et al., 2021)

HC (T0) also showed an inverse correlation with serum PROT (T1). Thus, the presence of leukopenia and anemia may indicate an initial process of immunosuppression and an increase in parasite load, which may lead to a change in hyperglobulinemia, an initial process of renal injury, and disease progression.

PH concentrations were also moderately correlated with UR and PTN levels. A study demonstrated an essential relationship between PH and proteinuria. When analyzing the renal protective response, it was found that for a reduction in proteinuria, a reduction in protein intake was not necessary, but a reduction in PH, either in its urinary excretion or serum concentration. Patients with low PH levels had the most significant decrease in proteinuria, regardless of UR excretion (a factor used to estimate protein intake) (Di Iorio et al., 2013).

Based on all findings and discussion it can be concluded that the most associated parameters are PL and SBP. In order to interpret the results and analyze the predictive factors and prognostic markers, we analyzed Figure 1 and isolated four patients who presented a negative evolution, starting from classification in stage IIa at T0 to stage IV at T1. Two patients developed thrombo-

cytopenia and systemic arterial hypertension at T0. Neither of the patients had proteinuria, azotemia, or hyperglobulinemia and had SDMA < 14μg/dL. The authors pointed out the importance of evaluating systemic hypertension and thrombocytopenia in dogs with *L. infantum* infection. The other two patients exhibited no changes in laboratory test results at T0. New studies should be carried out in order to evaluate PL and SBP as predictive factors and prognostic markers.

CONCLUSIONS

PL was the primary physiological biomarker to demonstrate value as a predictive factor and prognostic marker being related to biomarkers of kidney injury as PH and UPC. It has been shown that SBP, LE, HC, PH and UR. D are predictive or prognostic markers in dogs infected with *L. infantum.* There was no advantage of SDMA over UPC for assessing kidney injury in dogs infected with *L. infantum* at the time of the study. SDMA and UPC didn´t show significance as predictive or prognostic markers in dogs infected with *L. infantum*. It is concluded that the evaluation of thrombocytopenia is important in the evaluation of CanL, and its reversal corresponds to an improvement in the prognosis.

Author's Contributions

FSM and APCV: data collection, analysis and experimental design. They conducted the gathered the necessary data and performed statistical analysis on the results; assisted in data interpretation and manuscript preparation. They contributed equally to the interpretation of the experimental results, helped in drafting the manuscript, and critically reviewed the content for accuracy and clarity. JCCV and VMR contributed to the theoretical framework and literature review.

Competing interest

The author(s) declare that they have no competing interests.

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INFECTIOUS STOMATITIS IN CAPTIVE *SALVATOR MERIANAE* LIZARDS

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Abstract

Infectious stomatitis is a common ailment in captive reptiles, it arises from a combination of predisposing factors such as poor nutritional conditions, habitat issues or stress, where normal oral flora microorganisms act as pathogens, facilitating the disease development. This study aims to describe infectious stomatitis in a captive population of adult *Salvator merianae* lizards and to propose prophylactic measures for their maintenance in captivity. Within a population of 57 animals, a morbidity rate of 19.2% and a mortality rate of 7% were estimated. Microbiological analysis of oral mucosa revealed *Pseudomonas aeruginosa* susceptible to ceftazidime, ciprofloxacin, gentamicin, and amikacin. Symptoms ranged from asymptomatic to animals with mild oral cavity lesions or severe stomatitis, with some cases exhibiting respiratory complications. Histopathological examination of lung samples was consistent with caseous pneumonia. Intramuscular ceftazidime treatment and oral disinfection yielded excellent results for lizards with stomatitis, although a favorable response was not observed in animals with pneumonia.

Key words: Black and white tegu, caseous pneumonia, oral diseases, *Pseudomonas aeruginosa*, reptiles

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INFEKTIVNI STOMATITIS KOD GUŠTERA VRSTE *SALVATOR MERIANAE* KOJI SE DRŽE U ZATOČENIŠTVU

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Kratak sadržaj

Infektivni stomatitis je česta bolest kod gmizavaca koji se drže u zatočeništvu. Uzrokuje ga kombinacija predisponirajućih faktora kao što su loša ishrana, problemi sa staništem ili stres, gde normalni mikroorganizmi oralne flore deluju kao patogeni, što potpomaže razvoj bolesti. Ova studija ima za cilj da opiše infektivni stomatitis u populaciji odraslih guštera Salvator merianae koji se drže u kavezima i da izloži predloge profilaktičkih mera za njihovo držanje u kavezima. U okviru populacije od 57 životinja utvrđena je stopa morbiditeta od 19,2% i mortaliteta od 7%. Mikrobiološka analiza oralne sluzokože pokazala je prisustvo *Pseudomonas aeruginosa* koji je osetljiv na ceftazidim, ciprofloksacin, gentamicin i amikacin. Simptomi su bili raznoliki- od asimptomatskih životinja do onih koje su ispoljile simptome teškog stomatitisa, blage lezije usne duplje, a neke su imale i respiratorne komplikacije. Histopatološki pregled uzoraka pluća ukazao je na kazeoznu pneumoniju. Intramuskularni tretman ceftazidimom i oralna dezinfekcija dali su odlične rezultate za guštere sa stomatitisom. Međutim, nije primećen povoljan odgovor kod životinja sa pneumonijom.

Ključne reči: Crni i beli tegu, kazeozna pneumonija, oralne bolesti, *Pseudomonas aeruginosa*, reptili

INTRODUCTION

Adequate zootechnical management stands out as the most crucial factor for maintaining healthy reptiles bred in captivity. Inappropriate breeding conditions, such as overcrowding, nutritional deficiencies, infections, and parasitosis lead to stress-induced immunosuppression (Cobos and Ribas 1987; Meredith and Redrobe, 2012; Zhou et al., 2020; Tian et al., 2022). Numerous microorganisms constitute oral microbiota in reptiles. Nevertheless, disrup-

tions in the homeostasis of the immune response caused by the captive environment transform the commensal oral microbiota into opportunistic pathogens (Grego et al., 2017; Vega-Manriquez et al., 2018).

Infectious stomatitis, or "mouth rots," is a common oral mucosa infection in reptiles bred in captivity. While this disease has been predominantly described in snakes (Peñuela Gomez and Brieva Rico, 2007; Rojas-Sereno et al., 2015; Martins et al., 2021), it also affects turtles and some lizard species (Herrera Ramírez, 2008; Pereira et al., 2021). Symptoms of this pathology include sialorrhea, petechiae, plaques around the lips and mouth, facial malformations, gingival abscesses, and teeth loss. In cases that are not promptly controlled, the exudate from ulcerative stomatitis may be swallowed or aspirated, leading to gastroenteritis or bacterial pneumonia. In severe cases, the pathogen may also enter the general circulation, causing septicemia and death (Cobos and Ribas 1987; Mader, 2006; Meredith and Redrobe, 2012; Rodríguez Molano, 2015; Pereira et al., 2021; Rojas-Sereno et al., 2015; Doneley et al., 2018).

In saurians, pathological processes tend to be multifactorial, slow-progressing, and challenging to diagnose due to their inherent resistance and ability to mask symptoms. For this reason, in most cases, diseases are detected at advanced stages, which complicates the success of treatment and, in the case of captive populations, epidemiological control (Mader, 2006; Meredith and Redrobe, 2012).

This study provides a detailed report on the clinical manifestations, lesions, and treatment in a captive population of *Salvator merianae* lizards with infectious stomatitis.

MATERIALS AND METHODS

Animals

The study included 57 adult individuals from the *Salvator merianae* lizard breeding facility at the Facultad de Agronomía, Zootecnia y Veterinaria of the Universidad Nacional de Tucumán, province of Tucumán, Argentina (26° 51'S and 65° 17'W). The animals were housed in open-air enclosures with masonry fences, equipped with shelters containing dry grass and shade. For the welfare of adult *Salvator merianae* individuals, the minimum living space of 2 m^2, was provided (Manes, 2016). Ad-libitum feeding consisted of a diet specifically designed for this captive-bred species (Vega Parry and Manes, 2000). For individual health monitoring, radiofrequency identification devices (micro transponder ID-100, Trovan Electronic Identification, Rosenbusch, Buenos Aires, Argentina) were used.

All experiments, including all animal handling protocols, were carried out in accordance with the Principles of Laboratory Animal Care (National Institutes of Health, publication N° 85- 23, revised 1985), as well as specific national laws. All experiments were carried out and approved by the Ethics Committee of Consejo de Investigaciones de Universidad Nacional de Tucumán (CIUNT).

Characterization of pathogens

The samples from the buccal and gingival mucosa of animals exhibiting signs of advanced stomatitis were collected using swabs and transported and preserved in Stuart medium. Bacteriological culture and antibiogram testing were conducted using the agar diffusion method (Kirby-Bauer test).

Histopathology

Histopathological analysis of lung biopsies from recently deceased animals was performed using standard procedures, including fixation with 10% neutral buffered formalin, embedding, sectioning, and hematoxylin and eosin staining (Suvarna et al., 2018).

RESULTS

Epidemiology and clinical signs

The disease affected both males and females. Out of a total number of 57 adult animals that were examined, 4 exhibited symptoms of advanced stomatitis, 7 showed signs of mild stomatitis, and another 4 were cases with respiratory and/or systemic complications. The remaining 42 animals did not show any lesions in the oral mucosa indicative of clinical stomatitis. As population indicators, the morbidity rate of stomatitis with clinical signs was estimated at 19.2%, with a mortality rate of 7%.

The 4 individuals with the signs of advanced stomatitis had petechiae and ecchymosis in the oral mucosa, periodontitis, they experienced loss of dental pieces, and granulomatous plaques in the oral and lingual mucosa. In some cases, these signs were accompanied by oral, ocular, or nasal discharge (Figure 1A).

The seven animals that manifested mild signs of stomatitis, had inflammation of the oral and gingival mucosa, but without hemorrhagic lesions or any type of plaques.

The 4 animals with severe stomatitis and caseous necrosis in the mouth and tongue died due to respiratory and systemic complications. Only two of them showed evident signs of respiratory distress with dyspnea, white foamy expectorations, and loss of appetite. The other 2 did not show evident signs of respiratory complications, and the diagnosis was confirmed through post-mortem lesions (Figure 1B).

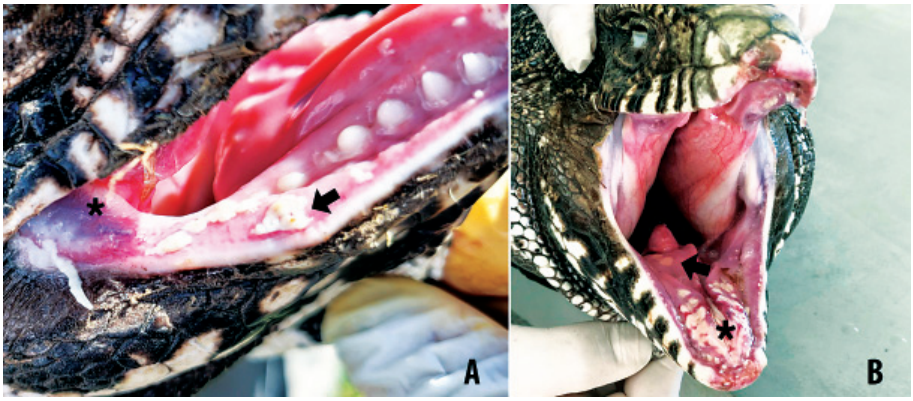

Figure 1. A. Adult female *S. merianae* lizard with stomatitis; arrow indicates granulomatous plaque on oral mucosa, asterisk indicates inflamed, congested, and ecchymotic area at oral commissure. B. Adult male *S. merianae* lizard with severe stomatitis; asterisk indicates apical region of the tongue and oral mucosa with caseous necrosis, arrow indicates granulomatous plaque on the body of the tongue.

Macroscopic findings

The necropsy of the recently deceased lizards revealed that the most affected organs were the lungs, although some macroscopic lesions were also found in the mouth, the fat bodies, and the liver. The fat bodies were notably congested and friable, with hemorrhagic lesions. Additionally, the friable liver had changed in its color. The lungs were the most affected organs, significantly hyperemic, and hemorrhagic with numerous pinpoint granulomatous plaques similar to those found in the oral cavity (Figure 2).

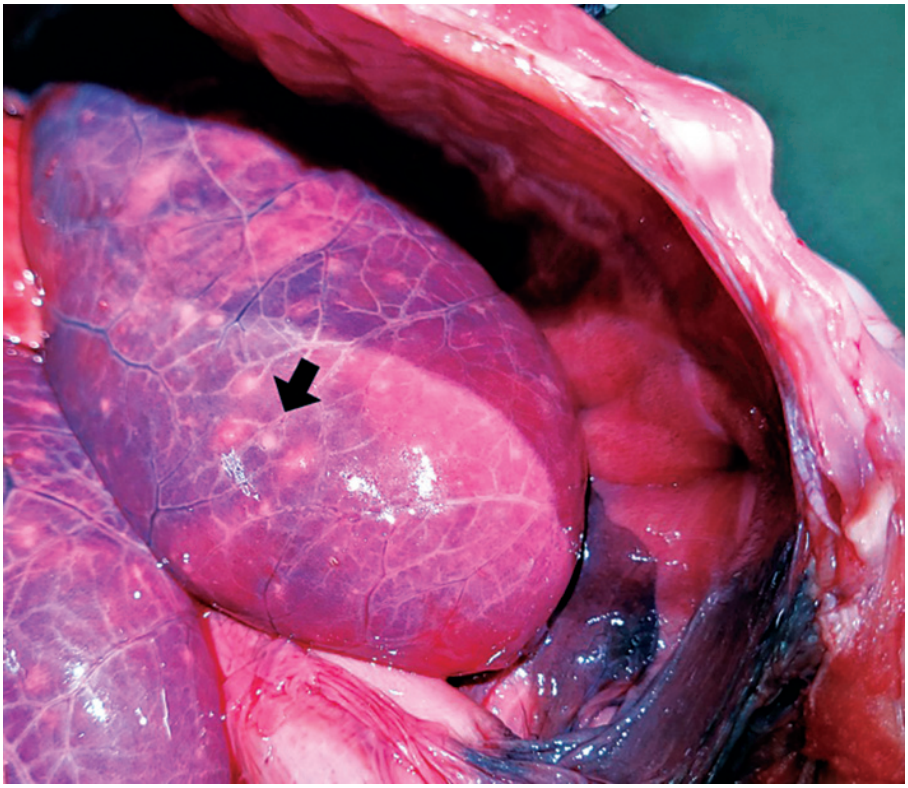

Figure 2. Lungs with numerous small granulomatous lesions (arrow).

Histopathology findings

In the submesothelial layer of the visceral serous tunic and the stroma of type III trabeculae in both lungs, inflammatory hyperplasia, characterized by the profusion of collagen fibers, was evident. Moreover, in the lumen of the pulmonary venules, there was a notable abundance of lymphocytes (Figure 3A). Necrotic lesions with infiltration of the pulmonary parenchyma and eosinophilic areas centrally located to the granulomatous lesion, composed of distinctive amorphous tissue of caseous necrosis, were also prominent (Figures 3B and 3C).

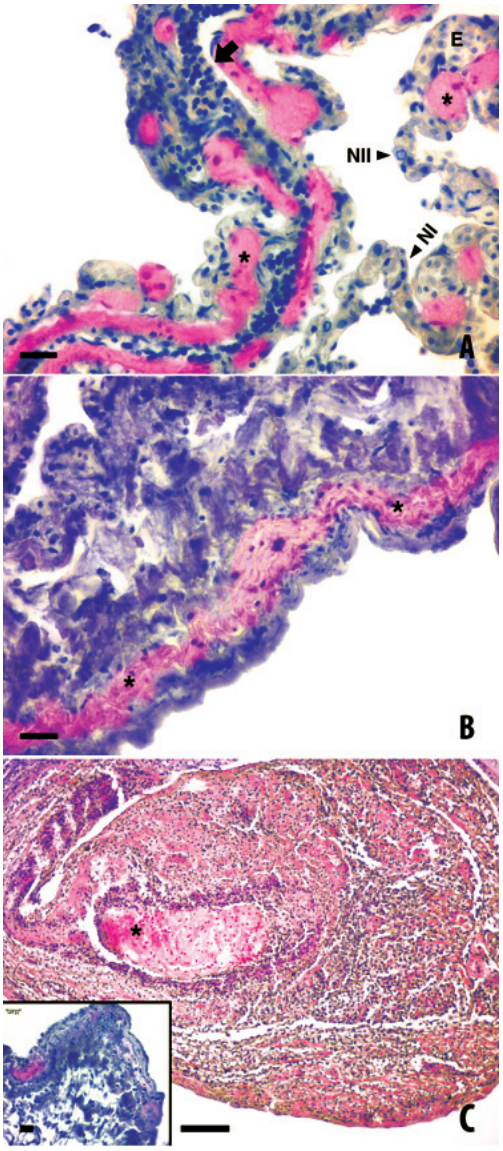

Figure 3. A. Detailed view of foveolar epithelium with marked hypertrophy of type III trabeculae (asterisks). Note the abundance of lymphocytes (arrow). NI, type I pneumocytes; NII, type II pneumocytes; E, erythrocytes. Scale bar 10 μm. B. Detailed view of visceral serous layer of the lung. Note the abundance of collagen fibers in the submesothelial layer (asterisks). Scale bar 10 μm. C. Lung section with granulomatous lesion (asterisk). Scale bar 50 μm. The insert shows characteristic necrotic tissue with absence of cellular boundaries and pyknotic nuclei. Scale bar 10 μm.

Culturing and antibiogram

Bacteriological culture of the oral mucosa of animals with the clinical disease revealed few Gram-positive cocci and few Gram-negative bacilli, as well as polymorphic nuclear cells. *Pseudomonas aeruginosa*, sensitive to antibiotics such as ceftazidime, ciprofloxacin, gentamicin, and amikacin, was isolated.

Treatment and evolution of animals with clinical signs

Based on the antibiogram results, intrinsic characteristics of each antibiotic (therapeutic margin, toxicity, duration of action, ease of application), and cost analysis, ceftazidime antibiotic therapy was chosen.

Animals with the signs of stomatitis with evident oral mucosa plaques and lesions underwent the following treatment: cleaning of wounds with 10 vol. hydrogen peroxide or 0.05% chlorhexidine and manual removal of oral plaques. Simultaneously, they were supplemented with 5000 IU of oral vitamin A once a week.

Depending on the characteristics of each patient and the severity of the clinical picture, between 3 and 6 applications of intramuscular ceftazidime at 30 mg/kg were performed every 72 hours until the reversal of symptoms.

In cases of mild or advanced stomatitis without respiratory complications, the treatment was highly effective, and the patient's evolution was favorable (Figure 4). However, in animals with clinical signs of pneumonia, the treatment was not effective.

Figure 4. Evolution of a stomatitis case: A and B. Initial state of oral wounds, left and right profiles, respectively. C. Oral wounds at 2 weeks after the start of treatment. Arrows indicate granulomatous plaques at both commissures, asterisk indicates petechiae. D. Resolution of oral wounds and patient's discharge 6 weeks after treatment initiation.

Population Management Measures

For metaphylactic treatment, the entire breeding stock received a single dose of 30 mg/kg of intramuscular ceftazidime. Weekly controls of the oral mucosa and body condition of 10 randomly selected animals were conducted for a month. When an individual showed any signs or suspected symptoms of stomatitis, it was isolated to start antibiotic treatment, vitamin A supplementation, and cleaning and disinfecting of the oral mucosa.

After a month, and in order to avoid stress from manipulation, inspections consisted of visual examination of animals in the corral, and only if suspicious, the animal was restrained for mucous membrane inspection. An animal was considered suspicious if it exhibited at least one of the following symptoms:

oral, ocular, or nasal discharge of any type, weight loss of body condition, or dehydration.

Sick or suspected animals were separated from the rest in an isolation area with the same shade, shelter, water, and ration conditions as the rest of the enclosures. Feeders, drinkers, and shelters throughout the breeding facility were disinfected with 0.1% sodium hypochlorite, and the substrate in all shelters was renewed.

DISCUSSION

The oral and intestinal microbiota varies significantly among different groups of reptiles and is influenced by their habitat, physiology, and nutrition. Generally, the oral flora in saurians is primarily composed of Gram-negative bacteria, such as *Escherichia coli, Salmonella, Pseudomonas, Proteus,* and *Aeromonas* (Meredith and Redrobe, 2012; Tian et al., 2020, 2022). These bacteria can be isolated from both healthy and diseased animals. It is known that healthy reptiles can transmit *Salmonella* or *P. aeruginosa*, representing a major zoonotic risk associated with keeping reptiles (Meredith and Redrobe, 2012; Martins et al., 2021). This is the first study reporting *P. aeruginosa* as a potential opportunistic pathogen causing infectious stomatitis in *S. merianae.*

The clinical signs observed in our animals coincided with those reported for stomatitis in other captive reptiles (Cobos and Ribas 1987; Mader, 2006; Meredith and Redrobe, 2012; Rojas-Sereno, 2015; Hedley, 2016; Doneley et al., 2018; Pereira, 2021). The lesions found in the oral cavity of diseased animals were similar to those described for *Boa constrictor amarali* with caseous stomatitis caused by *P. aeruginosa* (Martins et al., 2021). In reptiles, pneumonia signs often appear late, when the infection is chronic with significant respiratory involvement (Mader, 2006; Rodríguez Molano, 2015). In most cases observed in this study, the condition was limited to oral infection. However, in animals with pneumonia, macroscopic and histopathological lesions were consistent with chronic inflammatory processes, characterized by a marked increase in collagen fibers and notable lymphocytic infiltration. Timely treatment with intramuscular ceftazidime every 72 hours and oral antiseptics proved to be the appropriate combination for treating stomatitis in *S. merianae*. The combination of clinical examination, oral culture and antibiogram, histopathology, and favorable response to treatment, enabled us to confirm the diagnosis without the need for more expensive complementary tests.

The incidence of bacterial diseases in captive lizards is often associated with a compromised immune system resulting from overcrowding, trauma, nutritional deficiencies, infections, and excessively low temperatures that can

predispose normal microbial flora to act as opportunistic pathogens (Mader, 2006; Hedley, 2016; Van Zanten and Simpson, 2021). Regarding predisposing factors, overcrowding and poor management as promoters of stomatitis were ruled out. Indeed, we have optimized the zootechnical management of captive *S. merianae*, as well as the breeding conditions in which these lizards are kept and reproduced. This includes spacious enclosures with over 2 m^2/animal, shelters resembling their natural habitat, dirt floors for digging, and other elements of environmental enrichment (Manes, 2016; Van Zanten and Simpson, 2021). Although the animals are fed a diet specifically designed for this species (Vega Parry and Manes, 2000), maintaining a sustained mono diet for approximately 20 years, it is likely that they require supplementation of vitamins A, D, and E. Vitamin A deficiencies have been linked to stomatitis and inflammation of the upper respiratory tract (Herrera Ramírez, 2008; Rodríguez Molano, 2015). In this study, oral administration of vitamin A was chosen to coincide with the oral inspection of each patient, avoiding additional intramuscular injections that could be painful and stressful for the animal. Stress from increased frequency or duration of handling, including health management, predisposes the animals to illness and complicates the healing process (Doneley et al., 2018).

The immune response of reptiles is subjected to seasonal temperature-dependent alterations, with a maximum response observed when they are maintained near their preferred optimal body temperature (Mader, 2006; Doneley et al., 2018). Prolonged maintenance of reptiles at temperatures ranging from 15 °C to 21 °C predisposes them to opportunistic diseases. The innate and adaptive arms of the reptilian immune system are accepted to function optimally at the preferred body temperature of the animal. Many reptiles are kept at suboptimal temperatures, and simply warming them up can lead to significant improvements in their immunological defenses (Doneley et al., 2018). The monthly average temperature recorded in the region, where the breeding facility is located during the brumation period (May to September), was of 14.8 °C between 2018 and 2022 (EEAOC, 2023). This temperature would be at the limit of the suggested optimum for normal brumation (3.8 °C to 15 °C). Our results suggest that untimely winters with inappropriate temperatures for proper brumation may be a predisposing factor for stomatitis in captive *S. merianae*.

There is little knowledge about the immune system of reptiles. Some agents of reptiles are more or less infectious, but for most of them, the level of infectivity is unknown (Doneley et al., 2018). In our study, it is likely that *P. aeruginosa* acted as an opportunistic agent, and the great diversity of clinical

manifestations observed in our population may be attributed to the intrinsic immunity of each animal. The physiological state of each individual can impact susceptibility to diseases, even under similar captive conditions. Gravid females, males during breeding season, dominance of food sources, or other resources by enclosure mates, together with the effects of hierarchical stress, may predispose to illness (Meredith and Redrobe, 2012; Doneley et al., 2018).

CONCLUSIONS

This article provides a detailed report of clinical manifestations and macroscopic and histopathological findings in a captive population of *S. merianae* lizards with stomatitis. Identification of each individual facilitated precise treatment monitoring and the evolution of each patient. Furthermore, periodic follow-ups within the population have allowed us to suggest sanitary management measures to reduce the prevalence of this disease in captive animals.

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Author's Contributions

OLSL: Animal management, diagnosis and treatment of patients, drafting the manuscript. FHCC: Histopathological analysis, drafting the manuscript.

Competing interests

The author(s) declare that they have no competing interests.

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ECTOPIC PRIMARY ABDOMINAL PREGNANCY IN A PERSIAN CAT

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Abstract

Ectopic pregnancies are common in human medicine, while they are rarely recorded in animals. This report describes a case of 2.5-year-old ectopic primary abdominal pregnancy in a 7-year-old Persian cat. The cat was admitted to the surgery clinic for routine ovariohysterectomy with no characteristic clinical symptoms. During surgery, ovaries, uterus and ligaments were normal, like in a non-pregnant cat. Four intra-abdominal fetuses of different developmental stages were found and carefully removed. Three fetuses were found free in the abdomen inside their gestational sacs. The fourth fetus was covered with omentum and had no gestational sac. The gestational sacs had different sizes (2-8 cm in diameter). Two of the fetuses were fully-haired with normal position of limbs. No complications were recorded during the surgery and 10 months post-operative. This case report adds to the database of ectopic pregnancies in cats and further illustrates their incidental nature.

Key words: Abdominal pregnancy, cat, ectopic pregnancy, fetuses, ovariohysterectomy.

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VANMATERIČNI PRIMARNI ABDOMINALNI GRAVIDITET KOD PERSIJSKE MAČKE

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Kratak sadržaj

Vanmaterične trudnoće su česte u humanoj medicini, dok se retko beleže kod životinja. Ovaj izveštaj opisuje slučaj 2,5-godišnjeg vanmateričnog primarnog abdominalnog graviditeta kod 7-godišnje persijske mačke. Mačka je primljena na Kliniku za hirurgiju radi rutinske ovariohisterektomije bez karakterističnih kliničkih simptoma. Tokom operacije jajnici, maternica i ligamenti bili su normalni, kao kod mačke koja nije gravidna. Pronađena su i pažljivo izvađena četiri intraabdominalna fetusa različitih razvojnih stadijuma. Tri fetusa pronađena su slobodna u abdomenu unutar svojih gestacijskih vrećica. Četvrti fetus bio je prekriven omentumom i nije imao gestacijsku vreću. Gestacijske vreće bile su različite veličine (2-8 cm u promeru). Dva fetusa su bila sa potpuno formiranim dlačnim pokrivačem s normalnim položajem udova. Tokom operacije i 10 meseci nakon operacije nisu zabeležene komplikacije. Ovaj prikaz slučaja prestavlja dodatni podatak za bazu podataka o vanmateričnom graviditetu kod mačaka i dodatno ilustruje njihovu slučajnu prirodu.

Ključne reči: Abdominalni graviditet, mačka, vanmaterični graviditet, fetusi, ovariohisterektomija

INTRODUCTION

Ectopic pregnancy refers to a pregnancy occurring outside of the cavity of the uterus. While this disorder is common in humans, it is rarely recorded in animals (Vidiastuti et al., 2022). Ectopic pregnancies are classified according to the site of implantation and they can be abdominal and tubal pregnancies. The etiology and pathogenesis of ectopic pregnancy are not always clearly defined (Chong, 2017; Zheng et al., 2018; Jiasan et al., 2019).

Abdominal pregnancies are also subdivided into primary and secondary

forms. The primary form occurs when fertilized oocytes are released into the abdomen and implanted on the abdomen (Osenko and Tarello, 2014; Zheng et al., 2018; Vidiastuti et al., 2022). Secondary form occurs when a pregnant uterus is ruptured due to trauma or wounds and the fetuses are released into the abdominal cavity where they continue their development (Tirgari, 1986; Findik et al., 1998; Ivanova et al., 2019). Differentiation between primary and secondary extra-uterine pregnancies is controversial due to the presence of an intact (Dzięcioł et al., 2012), altered (Kumru et al., 2007) or partially missing reproductive system (Johnston et al., 1983). Consequently, the absence of signs of uterine rupture is one of the most important inclusion criteria for the diagnosis of primary extra-uterine pregnancies in cats (Osenko and Tarello, 2014).

Diagnosis of ectopic pregnancy is based mainly using X-ray (Johnston et al., 1983; Osenko and Tarello, 2014; Mirsepehr et al., 2015), ultrasound (Findik et al., 1998; Mirsepehr et al., 2015; Vidiastuti et al., 2022) and exploratory laparotomy (Bodle, 1979; Tirgari, 1986). Nevertheless, several cases of ectopic pregnancies were recorded in cats as an accidental finding during routine clinical examination or radiography (Mirsepehr et al., 2015; Chong, 2017; Ivanova et al., 2019). Moreover, the histological examination of the ectopic fetuses did not definitively prove their extra-uterine development (Rosset et al., 2011).

Death of the abdominal fetuses is a typical outcome of recorded ectopic pregnancies in cats due to insufficient nutrition resulting from a lack of adequate blood supply inside the abdomen (Mirsepehr et al., 2015; Chong, 2017; Zheng et al., 2018). Therefore, laparotomy, removal of the ectopic fetuses and ovariohysterectomy are usually performed for treatment and prevention of recurrence of ectopic pregnancies in cats (Mirsepehr et al., 2015; Chong, 2017; Zheng et al., 2018; Ivanova et al., 2019). Nevertheless, spontaneous resolution of early ectopic pregnancies may occur, suggesting that many diagnosed cases in early stages require no treatment at all (Mirsepehr et al., 2015; Chong, 2017).

Ectopic pregnancy in cats has been recorded in the veterinary literature. However, it is quite uncommon, as shown in Table 1. Therefore, this case report adds to the available database of ectopic pregnancies in cats and further explains their accidental nature.

Table 1. Types of ectopic pregnancies recorded in different breeds of cats and characters of the ectopic fetuses

Age/Breed	Type of ectopic pregnancy	Characters of the fetuses	Reference
A 3-year-old Persian cat	Primary abdominal pregnancy	One mummified fetus, 7.25 x 4.74 cm	Vidiastuti et al. (2022)
Two European shorthair cats	Secondary abdominal pregnancies	Three fetuses, 3 cm in diameter Two, 6 × 3.5 cm and 5 × 4.5 cm	Ivanova et al. (2019)
A British shorthair	Tubal pregnancy	One fetus, not identified	Jiasan et al. (2019)
Unidentified cat A British shorthair cat	Primary abdominal pregnancy Tubal pregnancy	One calcified fetus, 4 cm in diameter One immature fetus, unidentified size	Zheng et al. (2018)
A domestic, short-haired cat	Secondary abdominal pregnancy	Two fetuses, 10 cm from crown to rump	Chong (2017)
A domestic short-haired cat	Secondary abdominal pregnancy	Three fetuses, fully developed	Mirsepehr et al. (2015)
A domestic short haired spayed cat	Primary abdominal pregnancy	Three calcified fetuses, 4-5 cm in size	Osenko and Tarello (2014)
A domestic shorthair Unidentified cat	Secondary abdominal pregnancies	Two fetuses, different stages of development One outside the uterus and one inside, 30-35 days pregnancy	Dzięcioł et al. (2012)
A 1.5-year-old domestic short-haired cat	Secondary abdominal pregnancies	One mummified fetus, 7 cm in length	Rosset et al. (2011)
A 2-year-old crossbreed free-roaming cat	Secondary abdominal pregnancies	One dead fetus at 55 days' gestation	Kumru et al. (2007)
Angora cat	Secondary abdominal pregnancy	One fetus, 6 x 2.5 cm	Findik et al. (1998)
A 2.5- year-old short-haired cat	Secondary abdominal pregnancy	One encapsulated fetus, 7 x 4 x 4 cm and one embedded in the omentum, 7.5 x 3 cm	Tirgari (1986)

Age/Breed	Type of ectopic pregnancy	Characters of the fetuses	Reference
A 2.5- year-old domestic short-haired cat	Secondary abdominal pregnancy	Two necrotic fetuses, 7 x 4 cm	Johnston et al. (1983)
Unidentified cat	Primary abdominal pregnancy	Three mummified fetuses, 2,3 and 6 cm in diameter	Bodle (1979)

CASE PRESENTATION

This study was approved by the ethical committee at Faculty of Veterinary Medicine, Cairo University, Egypt. The cat was treated in accordance with guidelines established by the international and institutional Animal Care and Use Committees.

A seven-year-old Persian cat was brought to the veterinary clinic for routine ovariohysterectomy. The owner adopted the cat when it was 2.5 years old from the street. The cat showed normal sings of regular estrous cycles. Detailed history of previous pregnancies could not be obtained. During the preoperative examination, two large intra-abdominal hard masses were palpated in the middle abdomen and no other abnormalities were detected. The masses were freely mobile inside the abdominal cavity.

The cat was given general anesthetic for ovariohysterectomy and exploratory laparotomy. During surgery, three freely movable encapsulated intra-abdominal masses were found and easily removed. A fourth mass covered with omentum was also removed by careful blunt dissection. The ovaries, uterus and ligaments had normal appearance without any abnormalities. Ovariohysterectomy was carried out according to a standard procedure. Prior to closing, the abdominal cavity was thoroughly examined and washed with sterile warm normal saline solution. The cat was monitored for 10 months after surgery. A successful recovery without any complications was reported.

The dimensions of the removed masses were 8 x 5 x 4 cm, 6 x 5 x 3cm, 4 x 2 x 2 cm and 1.5 x 1 x 0.5 cm as shown in Figure 1. Three of these masses were covered with calcified sacs while the fourth mass was covered with omentum and had no sac. After opening the sacs of the three masses and removal of the omental tissue from the fourth mass, four mummified and calcified fetuses were found. Two fetuses were well-developed, fully haired and had normal positions of limbs (Figure 2). One of the well-developed fetuses had a normal body, while the second one had open abdomen without viscera as shown in Figure 2.

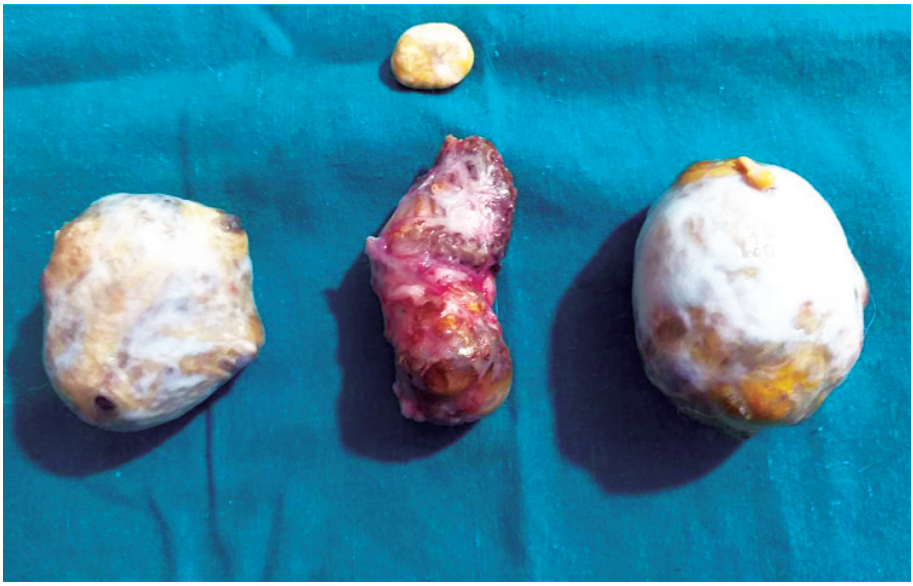

Figure 1. Four masses of different sizes were removed from the abdominal cavity of the cat. Note that three of them were encapsulated by gestational sacs and one embedded in the omentum.

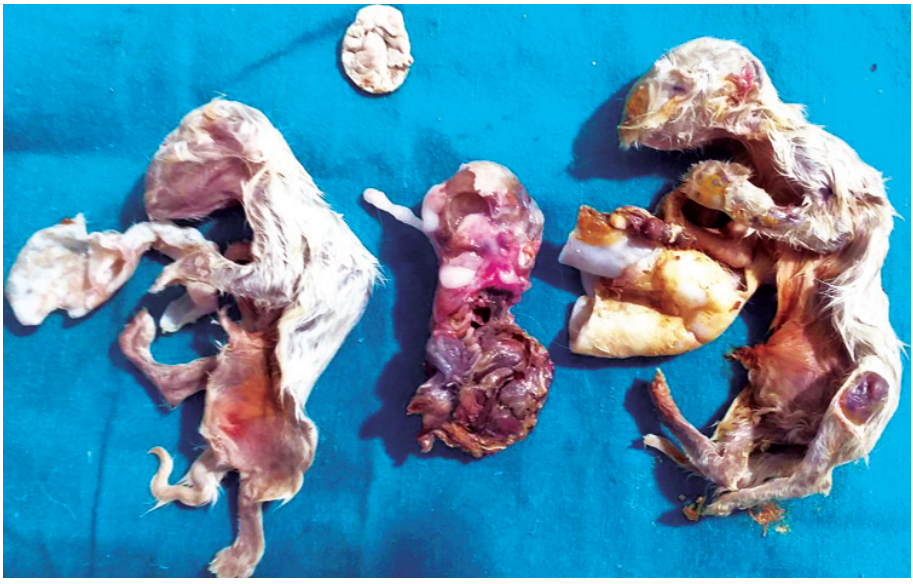

Figure 2. Four ectopic fetuses at different stages of development were identified after opening the gestational sacs and removal of the omentum.

DISCUSSION

While ectopic pregnancy is a common pathology in humans, it is still rarely recorded in veterinary literature. No detailed epidemiological studies on ectopic pregnancy have been conducted on animals, particularly cats. Cats are less likely to have ectopic pregnancy due to a difference in their endotheliochorial placentation (Zheng et al., 2018). This report describes a rare case of long-standing (> 2.5 years) ectopic primary abdominal pregnancy in a Persian cat.

Diagnosis of this case depended on the case history and findings of the clinical examination as well as laparotomy. In addition, abdominal ectopic pregnancy is truly primary when placentation presents onto a peritoneal or omental surface (Zheng et al., 2018; Vidiastuti et al., 2022), as in the current case. The differences between this case and other recorded ectopic pregnancy cases in cats are the number (N = 4) and different developmental stages of the ectopic fetuses as shown in Table 1.

Regarding the cat breed, domestic short-haired cats were the most commonly affected breed with ectopic pregnancies as shown in Table 1. However, the reported cat in this case study is a Persian cat. Similarly, a Persian cat with ectopic pregnancy was recently diagnosed by Vidiastuti et al. (2022).

Similar to the current cat, cats with ectopic pregnancy generally exhibit no clear clinical signs due to the aseptic condition of the ectopic fetuses which can remain within the animal's body for months or even years without complications (> 2.5 years). This is in agreement with the previously reported results by other authors (Dzięcioł et al., 2012; Zheng et al., 2018). Nevertheless, some cats with ectopic pregnancies, particularly secondary abdominal ectopic pregnancies, show various clinical signs such as loss of appetite, fever, vomiting, vaginal bleeding and peritonitis (Rosset et al., 2011; Dzięcioł et al., 2012; Mirsepehr et al., 2015). In other cases, particularly in primary abdominal ectopic pregnancies, cats have no clinical signs and they are detected during abdominal radiography or routine spaying as in the current case. Similar findings have been recorded before (Osenko and Tarello, 2014; Ivanova et al., 2019; Vidiastuti et al., 2022). Therefore, ectopic pregnancy may be detected as an incidental finding during clinical examination prior to ovariohysterectomy, like in the present case. The lack of associated clinical signs appears to demonstrate that such pregnancy is compatible with a normal healthy life in cats. Similar finding was confirmed by other authors (Chong, 2017; Zheng et al., 2018; Ivanova et al., 2019).

In the present case, only two of the four fetuses were palpable prior to the surgery. This could be attributed to the small size of the other two fetuses. This

finding is similar to the findings of other recorded cases of ectopic pregnancies in cats (Dzięcioł et al., 2012; Osenko and Tarello, 2014; Zheng et al., 2018; Ivanova et al., 2019). Therefore, radiography and ultrasonography examinations are useful tools for diagnosis of this problem in cats (Osenko and Tarello, 2014; Zheng et al., 2018). The main limitation in the present case report is the lack of radiography and ultrasonography examinations due to their unavailability at the time of examination.

During surgery, the uterus, ovaries and ligaments were normal in the present case. Therefore, it was diagnosed as primary abdominal pregnancy. Similar findings were recorded by other authors (Zheng et al., 2018; Ivanova et al., 2019). Unlike in these findings, ectopic abdominal fetuses secondary to trauma were observed in cats with multiple uterine abnormalities (Johnston et al., 1983; Dzięcioł et al., 2012; Chong, 2017). Moreover, Zheng et al. (2018) and Jiasan et al. (2019) diagnosed tubal ectopic pregnancy in the fallopian tubes of cats, and Osenko and Tarello (2014) recorded a case of ectopic pregnancy in a spayed cat at necropsy.

In the current case, laparotomy, removal of the ectopic fetuses and ovariohysterectomy were performed for treatment and prevention of recurrence of ectopic pregnancy. The surgeries were simple and without any early or late complications. This can be explained by the lack of attachment of the fetuses to any of the internal organs. These findings are in accordance with the findings of other authors (Mirsepehr et al., 2015; Chong, 2017; Zheng et al., 2018; Ivanova et al., 2019).

The number of extra-uterine fetuses ranged between one and three as shown in Table 1. The cat had four extra-uterine fetuses. According to the available literature, this is the largest number of extra-uterine fetuses recorded in cats to this day. In addition, the removed fetuses here were grown to different stages of development. The difference in developmental stages of the removed fetuses can be explained by the difference in the time of death of each fetus. Therefore, there was a variation in the size of the removed ectopic fetuses. Development of abdominal fetuses to advanced stages without an elaborated placentation has been recorded previously in cats (Mirsepehr et al., 2015).

According to Knospe (2002), the removed ectopic fetuses died between three and eight weeks of gestation. At 60 days of gestation, pigmentation can occur on the skin, hair, and nails, the brain and bones ossification also are present at this time. Signs of fetal mummification and calcification were observed in all ectopic fetuses. This is the common end of all recorded cases of ectopic pregnancies in cats due to malnutrition of the ectopic fetuses (Mirsepehr et al., 2015).

CONCLUSION

This is an interesting rare case of long-lasting ectopic primary abdominal pregnancy in a Persian cat. Four extra-uterine fetuses at different stages of development were present inside the abdomen for more than 2.5 years without any clinical signs or complications.

Competing interest

The author declares no conflicts of interest affecting the work reported in this paper. This research received no external funding.

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