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ORIGINAL ARTICLE

Decontamination protocols for bovine fecal and environmental samples for culture of *Mycobacterium avium* subsp. *paratuberculosis* growth on solid media

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ABSTRACT. Bacteriological culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is considered the gold standard to confirm its presence in several matrices for Johne's disease diagnosis. Whether it is a liquid or solid culture, a problem with MAP culture is that non-interpretable results arise because of overgrowth by other microorganisms, making MAP growth and identification more difficult or impossible. We systematically reviewed published decontamination protocols and their effects on MAP culture from bovine fecal and environmental samples on solid media. Based on our findings, we suggest a step-by-step decontamination protocol. The OVID®/MEDLINE, PubMed®, SciELO Citation Index®, and Redalyc® platforms as well as the International Colloquium on Paratuberculosis (ICP) proceedings and the reference lists were reviewed to identify relevant studies. The inclusion criteria considered articles published in English, Portuguese, French, German, Spanish, and peer-reviewed journals. The exclusion criteria included unrelated topics, species other than bovines, other than environmental/faecal samples, other than diagnostic techniques of interest, and non-original articles. Definitive studies were obtained through the authors' consensus regarding their eligibility and quality. In total, 1,004 publications matched the search terms, and 27 articles met the inclusion criteria, of which 45 derived and reported 15 different decontamination protocols. The centrifugation-one-step hexadecylpyridinium chloride protocol, which used over 22,154 fecal samples in three studies, was found to be the most suitable, reporting an average MAP isolation rate of 3.99% (886/22,154) and an average contamination rate of 0.17% (38/22,154). This systematic review highlights the need for further refinement of decontamination protocols to minimize the loss of viable MAP during processing of bovine fecal and environmental samples.

Keywords: Cattle, culture, HEYM, Johne's disease, overgrowth, paratuberculosis.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow-growing, mycobactin-dependent, acid-fast bacterium that causes paratuberculosis (PTB), a slow-developing, incurable cattle disease (Sweeney, 1996). MAP infection is characterized by chronic granulomatous enterocolitis, which occurs after a long and variable incubation period (Clarke, 1997; Harris & Barletta, 2001). This disease causes significant economic losses in infected herds (Nielsen & Toft, 2011), such as decreased milk production, decreased slaughter value, and premature culling. The estimated loss varies from 6 to 19% in the production of meat, milk, or both (McAloon *et al*., 2016; Shephard *et al*., 2016), and the average annual losses in major dairy-producing regions worldwide have been estimated at US$33 per cow, or ~1% of gross milk revenue (Rasmussen *et al*., 2021). In addition, zoonotic potential has been proposed since MAP has been frequently found in humans with Crohn's disease (Eltholth *et al*., 2009; Waddell *et al*., 2015).

Worldwide disease control is based on herd testing and strategic changes in herd management practices (Field *et al*., 2022). One difficulty in PTB control is that animals are infectious before being clinically infected or diseased (subclinically infected animals) (Fecteau & Whitlock, 2010). Moreover, subclinically infected animals may not be 100% detected using the available diagnostic tests (Sweeney *et al*., 2012).

Several tests are available for MAP diagnosis. These include tests for the pathogen's detection (culture or direct PCR of feces, tissues, or milk), tests for the host's immune response (antibody detection ELISA on serum or milk, various assays for cell-mediated immunity such as delayed-type hypersensitivity tests), or tissue inflammatory response (gross pathology and histopathology) (Nielsen & Toft, 2008; Stevenson, 2010). The sensitivity and specificity of tests for the diagnosis of PTB vary significantly depending on the MAP infection stage and intrinsic characteristics of each test. Sensitivity estimates for the bacterial culture of MAP from feces range from 16 to 74% across species and stages of disease (Nielsen & Toft, 2008; Whittington *et al*., 2017), and its specificity is considered to be almost

100% if a confirmation test, such as polymerase chain reaction (PCR), is used to confirm MAP isolation (Tavornpanich *et al*., 2008; Whittington *et al*., 2011).

MAP can be cultured on either liquid or solid media. Liquid culture methods have a higher analytical and diagnostic sensitivity than solid medium, and growth can be detected sooner, but a formal identification of MAP by a molecular method is required, making the identification of MAP more difficult and expensive (Eamens *et al*., 2000; Whittington, 2009; Whittington *et al*., 2017). However, identification of the organism is more difficult in liquid culture because the appearance of colonies and mycobactin dependence are not observable, and the growth of other organisms needs to be ruled out. In addition, such organisms are more sensitive to recovery than MAP C strains because the ability of solid media to support their growth is well established (Whittington, 2009).

The MAP microbiological culture process is based on the general principles of mycobacterial culture procedures: 1) decontamination to reduce the number of other microorganisms; 2) incubation in a culture medium that promotes MAP growth; 3) recognition of MAP colonies on solid media or a particular sign of growth in liquid media; and 4) MAP identification either by phenotypic or genotypic methods (Merkal *et al*., 1964; Dane *et al*., 2022).

Culture contamination can be due to the light growth of irrelevant microorganisms (IMs), a MAP-mixed culture, or to a complete overgrowth of the medium, hindering the growth and identification of MAP. However, precautions must be taken to prevent contamination by IMs, as this can reduce the diagnostic sensitivity of culture and increase the complexity and cost of confirming the presence of MAP (Whittington, 2009; Dane *et al*., 2022). Such scenarios reveal the importance of defining a decontamination process that is sufficient to prevent IMs and allow MAP detection. Moreover, a significant amount of dispersed and relatively inconsistent information on MAP decontamination protocols and their effect on the excessive growth of IMs is available worldwide. Therefore, this study aimed to systematically review the literature on decontamination protocols and their effect on the excessive growth of IMs or contamination in solid media used in the bacteriological culture of MAP from bovine fecal and environmental samples, and to suggest a decontamination protocol, according to our results.

MATERIAL AND METHODS

This systematic review (SR) was designed, performed, and reported in accordance with the PRISMA guidelines, as suggested by Page *et al.* (2021). An a priori established and pre-tested SR protocol was carried out, including the study question, procedure for literature search, study inclusion/exclusion criteria, checklists for conducting relevance screening, basic characterization, methodological assessment, and data extraction on relevant primary research.

Throughout this SR, we refer to a citation or article (depending on the phase of the systematic information analysis process) as an entire paper, publication, or research report, and to a study as the group of samples collected with a particular purpose within the citation or article.

Search strategy

The primary search was conducted on December 10, 2022. The process of identifying relevant articles considered a specific research question: How do decontamination protocols affect the growth of microorganisms other than MAP-irrelevant microorganisms in solid media cultures in bovine fecal and environmental samples? Four databases were searched (i.e., OVID®/MEDLINE, PubMed®, SciELO Citation Index®, and Redalyc®). The proceedings from the 3rd (1991) to the 12th (2014) International Colloquium on Paratuberculosis (ICP) were available from the platforms explored and therefore reviewed. The 13th and 14th proceedings (2016 and 2018, respectively) were available on the International Association for Paratuberculosis website. This last material was hand-searched for existing published primary studies. In addition, references related to the SR subject were manually searched in Behr & Collins (2010) and Behr *et al.* (2020) books to track primary publications, as well as in two previous reviews on the topic (Dane *et al*., 2022; Field *et al*., 2022).

The topic was divided into components, and the search terms used to find relevant studies on the platforms were (*mycobacterium avium paratuberculosis* OR paratuberculosis OR johne*) AND (cattle OR bovine? OR cow OR dair* OR beef OR heifer? OR bull? OR calf OR calves OR environment* OR livestock) AND (faec* cultur* OR fecal cultur* OR bacteri* cultur* OR bacteri* isolat* OR microb* cultur* OR microb* isolat* OR environment* cultur* OR cultivation) AND (contamina* OR decontamina* OR irrelevant? OR microorganism? OR irrelevant bacteri* OR fung* grow* OR overgrow*).

Eligibility screening

The inclusion criteria considered only original articles published in English, Portuguese, French, German, and Spanish, and in peer-reviewed journals. No publication year or country limitation was considered. In the case of ICP proceedings and other abstracts found, studies were included in the search strategy only to identify further citations already published in peer-reviewed journals. The first selection of publications was performed according to the information contained only in the title. Two of the authors performed the selection, and the Kappa coefficient was estimated. The inclusion of citations was performed considering the possibility of answering our investigation question. The reasons for not inclusion were as follows: i) irrelevant topics (e.g., coronavirus, *Staphylococcus*, *Salmonella*, chemotherapy, Crohn's disease, economic impact, control programs, *Mycobacterium bovis*, vaccine); ii) other-than bovines (e.g., goats, sheep, human, pigeon); iii) other-than environmental/fecal

samples (e.g., milk, tissue, cheese); iv) other-than diagnostic technique of interest (e.g., liquid media, PCR, ELISA, RFLP, radiometric culture, AGID, PMS-phage assay); v) not an original article (e.g., review, book). Duplicated articles were not considered. All the citations selected by at least one of the three authors were considered to continue the process.

Two authors screened eligible articles using the abstract. The Kappa coefficient was then calculated. The inclusion and exclusion criteria were the same as those for title screening. Conflicting was resolved through consensus between reviewers and if necessary, by a third reviewer. The remaining articles were studied by two authors using the entire paper to ensure that they contained relevant information (evidence) to answer the question. The Kappa coefficient was then calculated. Each full text was reviewed with particular attention to the materials and methods, and results sections. Articles were considered eligible if the following criteria (in addition to the conditions mentioned above for title and abstract screening) were assessed: 1) describes or cites a primary source of the decontamination protocol(s), and 2) reports contamination rate. Conflicting was resolved through consensus between reviewers and if necessary, by a third reviewer.

Two authors manually searched the reference lists of relevant articles identified by full-text screening for additional published primary articles (snowballing).

ICP proceedings and other abstracts identified during the primary search were revised to identify further citations in peer-reviewed journals. In this regard, abstracts that were able to answer the research question were identified, and an email was sent to the corresponding author (or other available) to inquire if the study related to such an abstract had been subsequently published in a peer-reviewed journal. The articles obtained from this step, as well as those detected by Behr & Collins (2010) and Behr *et al*. (2020), were screened by two of the authors.

Data extraction

After all available articles were compiled, a descriptive summary was prepared in the form of a large summary table, taking into consideration bibliometric information, decontamination protocol, contamination rate, MAP detection rate, and other relevant findings about the question of interest.

RESULTS

The electronic search, combining results from both search engines and removing duplicate references, yielded 1,004 eligible citations potentially related to the subject of this SR. The 13th and 14th proceedings (2016 and 2018, respectively) provided 17 abstracts found by hand searching, but none progressed to the next phases of the SR. Citations to be screened were published between 1949 and 2022.

After reading the titles, 764 were considered irrelevant (consented by two authors). The final number of citations obtained by title screening was 240 (retained by at least one reviewer). After reading the abstracts of the articles, 78 were excluded (by both authors) and 162 original articles remained for full-text review. Twenty-seven articles were completely reviewed by full-text and kept for data extraction, after dismissing 96 articles because the decontamination protocol and/or contamination and/or MAP-recovery rates were not available in sufficient detail.

The snowballing strategy was then applied to the reference lists of the 27 definitive articles, and no more citations were found. In addition, the same strategy was applied to books and reviews, and no more citations were found. The final number of articles fulfilling the eligibility criteria and hence included in the qualitative synthesis was 27. Figure 1 describes the SR protocol and the selection of relevant articles.

All articles were written in English, except for one each in German and Spanish. The first relevant publication was published in 1972 and the most recent in 2017. Relevant citations were published in 21 journals.

Table 1 presents the general information extracted from the relevant articles concerning the research question, including results in terms of overgrowth of the solid media used and MAP-recovery. A total of 45 studies were derived from the relevant articles, reporting the use of 15 different decontamination protocols applied to 41,847 bovine fecal and environmental samples cultured in solid media, considering each pool as an individual sample, because the estimates of contamination and recovery of MAP are also individualized.

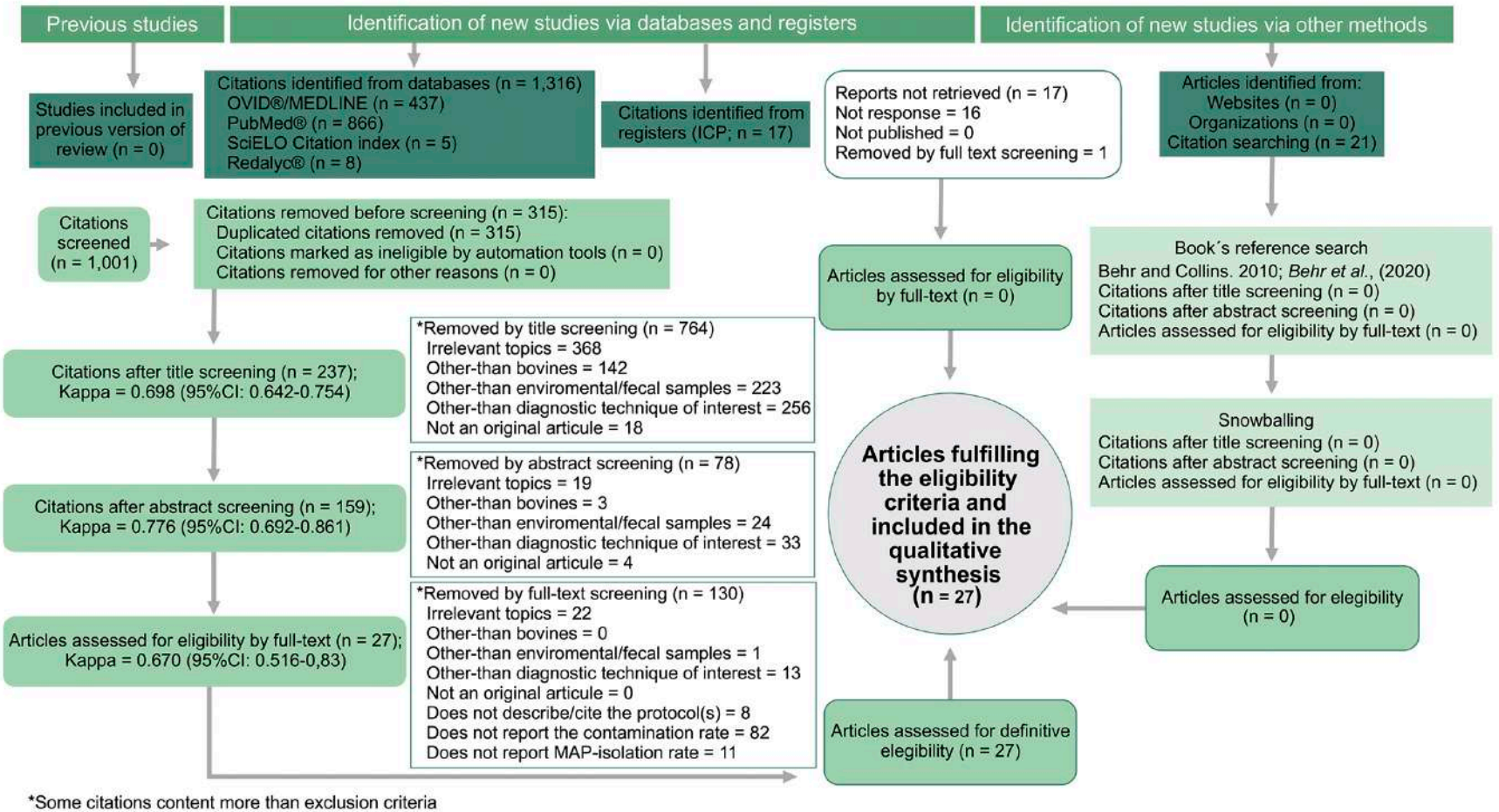

Figure 1.
Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart describing the progress of citations in a systematic review.

Table 1.
General information extracted from relevant articles concerning the research question.

Reference	Decontamination protocol (source, if reported)	Study number	Number of samples+	MAP-isolation rate (%)	Final contamination rate (%)
Merkal & Richards (1972)	Sedimentation-one-step BAC	Study 1	788 (I)	5.5	13.7
Jorgensen (1982)	Sedimentation-centrifugation-one-step NaOH/OA (Beerwerth 1967)	Study 1	1,413 (I)	31.9	15.4
		Study 2		41.7	2.6
Kim *et al.* (1989)	Sedimentation (Whipple & Merkal 1983)	Study 1	131 (I)	43.8	25.9
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)			49.6	60.3
	Sedimentation (Whipple & Merkal 1983)	Study 2		NR*	25.9
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)				60.3
	Sedimentation (Whipple & Merkal 1983)	Study 3		38.8	27.5
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)			44.6	37.4
	Sedimentation (Whipple & Merkal 1983)	Study 4		NR**	27.5
	Centrifugation-one-step HPC-water (Turcotte *et al.* 1986)				37.4
McNab *et al.* (1991)	Centrifugation-one-step HPC-water	Study 1	2,943 (I)	0.13	19.2
Whipple *et al.* (1992)	Sedimentation-one-step HPC-water (Cornell method)	Study 1	170 (I)	16.7	5.3
	Centrifugation-one-step HPC	Study 2		18.8	15.9
	Double-incubation-centrifugation-one-step HPC (Cornell method)	Study 3		15.9	0
Stabel (1997)	Sedimentation-one-step HPC-water	Study 1	24 (I)	56.5£	75
				60.9 ££	
	Centrifugation-one-step HPC	Study 2		47.8£	100
				41.7££	
	Double-incubation-centrifugation-one-step HPC (Cornell method)	Study 3		45.8£	0
				51.2 ££	
	Double-incubation-centrifugation-one-step HPC (NADC method)	Study 4		66.7£	20
				83.3££	
Reichel *et al.* (1999)	Sedimentation-centrifugation-one-step HPC-water	Study 1	450 (I)	23	5.8
McDonald *et al.* (1999)	Sedimentation-one-step HPC	Study 1	168 (I)	0	4
	Double-incubation-double centrifugation-one-step HPC-water (modified from Whitlock & Rosenberger 1990)	Study 2	210 (I)	6.2	16
Kalis *et al.* (1999)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	733 (I)	5.9‡	22,6
				63.6‡‡	
		Study 2	151 (P)	18.5‡	19.9
				72.7‡‡	
Kalis *et al.* (2000)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	2,989 (I)	5	7,04
	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 2	59 (I)	72.9†	0
				78††	
Eamens *et al.* (2000)	Sedimentation-one-step HPC-water	Study 1	179 (I)	8.4	0
	Double-incubation-centrifugation-one-step HPC-water (Whitlock & Rosenberger 1990)	Study 2		14.5	0.6
Soto *et al.* (2002)	Double-incubation-centrifugation-one-step HPC-water	Study 1	250 (I)	16	7.6
Nielsen *et al.* (2002)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	3,125 (I)	4.6	14.8

Reference	Decontamination protocol (source, if reported)	Study number	Number of samples+	MAP-isolation rate (%)	Final contamination rate (%)
Huda *et al.* (2003)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	371 (I)	4	23
Muskens *et al.* (2003)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	422 (I)	17.3	16.5
Sorensen *et al.* (2003)	Double-incubation-centrifugation-one-step HPC-water (Stabel 1997)	Study 1	500 (P)	3.4	6
Glanemann *et al.* (2004)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	1,144 (I)	8.1	17.6
	Double-incubation-centrifugation-one-step HPC (Shin 1989; Whitlock & Rosenberger 1990)	Study 2		1.6	21.5
Nielsen *et al.* (2004)	Sedimentation-centrifugation-NaOH/OA (Beerwerth 1967)	Study 1	2,513 (I)	3.3	13.2
		Study 2		6.9	14.6
Soumya *et al.* (2009)	Double-incubation-centrifugation-one-step HPC-water (OIE 2004)	Study 1	40 (I)	52.5	10
Gao *et al.* (2009)	Double-incubation-centrifugation-one-step HPC (Whipple *et al.* 1991; Stabel 1997)	Study 1	110 (I)	41.8	0.9
Fernández-Silva *et al.* (2011)	Centrifugation-one-step HPC (according to FLI 2007)	Study 1	36 (P)	5.7	8.6
		Study 2	1 (P)	100	0
Laurin *et al.* (2015)	Double centrifugation-one-step HPC (Stabel 1997)	Study 1	345 (I)	45.6	9.3
Donat *et al.* (2015)	Centrifugation-one-step HPC (according to FLI 2012)	Study 1	200 (I)	14.5	14
Donat *et al.* (2016)	Centrifugation-one-step HPC (according to FLI 2012)	Study 1	22,057 (I)	4	0.07
Heuvelink *et al.* (2017)	Centrifugation-one-step HPC (according to FLI 2010)	Study 1	61 (I)	1.6	30
	Sedimentation-centrifugation-NaOH/OA (Kalis *et al.* 1999; Rothkamp *et al.* 2009)	Study 2[ǂ]		5.6	5
		Study 3[ǂǂ]		8.2	5
Correa-Valencia *et al.* (2017)	Sedimentation-centrifugation-one-step HPC	Study 1	27 (P)	0	7.4
Noll *et al.* (2017)	Sedimentation-centrifugation-one-step HPC	Study 1	237 (I)	7.2	5.5

MAP, *Mycobacterium avium* subsp. *paratuberculosis*; BAC, benzalkonium chloride; NaOH, sodium hydroxide; OA, oxalic acid; HPC, hexadecylpyridinium chloride; NADC, National Animal Disease Center; FLI, Friedrich-Loeffler-Institut-Amtliche Methodensammlung (Official Collection of Methods). (http://www.fli.bund.de/fileadmin/dam_uploads/Publikationen/Amtliche_Methodensammlung/Methodensammlung_201204.pdf; http://www.fli.bund.de/fileadmin/dam_uploads/Publikationen/Methodensammlung_2010-07-07.pdf); NR, not reported.

[+] Cultured in pool (P) or individual (I)

[*] MAP-isolation rate not reported because it was a control of Study 1

[**] MAP-isolation rate not presented because it was a control of Study 3

[£] 1 g-sample

[ff] 2 g-sample

[‡] At cow-level

[‡‡] At herd level

[†] Low speed centrifugation (1,000×g for 15 min)

[††] High speed centrifugation (3,000×g for 15 min)

[ǂ] Cultured on Herrold's egg yolk medium (HEYM)

[ǂǂ] Culture on Lowenstein-Jensen medium

The MAP isolation rate from both bovine fecal and environmental samples ranged from 0 to 100% and the same for the contamination rate. The three main protocols, according to the number of fecal samples analyzed (n = 36,432) and the studies included (20/45), corresponded to 84.6% of the total samples included in this SR. These protocols are presented according to the number of fecal samples evaluated. The centrifugation-one-step HPC protocol was used for over 22,154 fecal samples from six studies. The average MAP isolation rate for this protocol was 3.99% (886/22,154), ranging from 1.64 to 5.7%; and the average contamination rate was 0.17% (38/22,154), ranging from 0.07 to 30%. The sedimentation-centrifugation-NaOH/OA protocol was used for over 12,830 fecal samples in nine studies. The average MAP isolation rate for this protocol was 8.57% (1,100/12,830), ranging from 3.3 to 78%, while the average contamination rate was 19.9% (2,554/12,830), ranging from 0 to 23%. Double-incubation-centrifugation-one-step HPC was used for over 1,448 fecal samples in five studies. The average MAP isolation rate for this protocol was 8.14% (118/1,448), ranging from 1.6 to 83.3%, while the average contamination rate was 17.4% (252/1,448), ranging from 0 to 21.5%. The most commonly used solid medium was Herrold's egg yolk medium (HEYM) (26/45).

From here and according to the decontamination protocols described by each paper, the frequency was as follows (from highest to lowest): the centrifugation-one-step HPC-water protocol, the double-incubation-centrifugation-one-step HPC-water protocol, and the sedimentation protocol were reported by four of the relevant articles each one; the sedimentation-one-step HPC-water protocol was reported by three of the relevant articles; the sedimentation-centrifugation-one-step HPC was reported by two of the relevant articles; finally, the centrifugation-one-step HPC-water protocol, the double centrifugation-one-step HPC protocol, the double-incubation-double centrifugation-one-step HPC-water protocol, the sedimentation-one-step BAC protocol, the sedimentation-one-step HPC protocol, the sedimentation-centrifugation-one-step HPC-water protocol, and the sedimentation-centrifugation- one-step NaOH/OA protocol were reported by one of the relevant articles each one. All previous results are based only on single data reports (i.e., individual, pool).

DISCUSSION

The aim of this SR was to collect, describe, and analyze studies reporting the effects of decontamination protocols on solid media culture results when complex matrices such as feces and environmental samples were processed for MAP detection, allowing us to answer the research question of how decontamination protocols affect the growth of microorganisms other than MAP-irrelevant microorganisms in solid media cultures in bovine fecal and environmental samples. This question is important because microbiological contaminants have been shown to inhibit the growth of MAP or to hide MAP colonies in solid media (Secott *et al*., 1999; Whittington, 2009). Thus, contaminants complicate, delay, and increase the cost of MAP culture.

Questions arising from this report are not intended to extend to liquid media, other than bovine samples, and other-than-fecal and environmental sample cultivation. The scope of our SR, defined at the protocol-definition stage, considered a solid culture of environmental and bovine fecal samples, since the technique and matrices are now considered routine procedures and are available from certain diagnostic laboratories. In addition, there is evidence from several laboratories that solid media are cheaper, less instrumentation is required, and identification of the organism is simpler (Whittington, 2010; Dane *et al*., 2022). Regardless of the analysis matrix, there is always a need to control the contamination and consequently the IM-related results, which apparently affect more liquid cultures compared to solid cultures (Whittington, 2009, 2010). Moreover, when the definition of the strain (MAP-type) is considered, it cannot be assumed that all strains of MAP have the same or similar antimicrobial resistance patterns, so each combination of antibiotics used both during decontamination procedures or within culture media (solid or liquid) will need to be carefully evaluated for each MAP type (Whittington, 2009).

Considering our methodology, the databases allowed access to information from 1910 to the present. Since the first report of consistent solid-media cultivation of MAP was published in 1912 (Twort *et al*., 1912), many variations of the original culture methods have been explored and implemented. Nevertheless, it should be noted that advances in the culture of MAP from fecal and environmental samples have been delayed by the distinctive growth characteristics of this bacterium. In addition, the need for a prolonged incubation period to culture the organism has led to numerous studies searching for a proper combination of decontaminants and antimicrobials that can effectively inhibit bacterial and fungal contaminants without affecting the growth of the primary isolate (Merkal & Curran, 1974; Jorgensen, 1982; Whitlock & Rosenberger, 1990; Stabel, 1997; Gwóźdź, 2006; Whittington, 2009).

Considering the relationship between the effectiveness of decontamination and the success of MAP isolation, we reported both contamination and MAP isolation rates. Both should be included in the methodological assessment of MAP-related studies (Stabel, 1997; Nielsen *et al*., 2004). Several reasons for the decontamination steps may explain the variability in the reported estimates (both contamination and MAP recovery rate) from solid cultures aimed at detecting MAP.

No differences related to sample quantity were reported or mentioned by any of the authors of the relevant studies. Nevertheless, the amount of matrix cultured (i.e.,

feces, environmental sources) should be standardized, as other researchers have reported to influence the contamination rate. Only to mention one case, Stabel (1997) recommended using 2 g of fecal samples rather than 1 g along with the Cornell decontamination method (double incubation-centrifugation) on HEYM culture to improve detection of subclinically MAP-infected animals.

Different methods for reducing bacterial and fungal contamination, including oxalic acid (OA), sodium hydroxide (NaOH), sodium hypochlorite, phenol, benzalkonium chloride (BAC), and hexadecylpyridinium chloride (HPC), have been evaluated for the isolation of MAP, emphasizing the latter two chemical decontaminants (Stabel, 1997). The cationic quaternary ammonium compound HPC was first used in the USA, replacing BAC (Merkal, 1984), and is now the basis for recent protocols in many countries, including North and South America, Europe, and Australasia. Eamens *et al.* (2000) concluded that longer double incubation times (24 and 48 h) in HPC and a mixture of amphotericin B, nalidixic acid, and vancomycin (VAN) were positively related to lower contamination. Mokresh *et al.* (1989) also found a reduction by about 2 log10 after exposure to 0.75% HPC for 18 h. However, other authors have reported no effect of HPC for up to 5 days (Whipple *et al.*, 1992). Progressive loss of viable organisms occurs with each step-in sedimentation and centrifugation protocol, as only part of the material from one step is taken forward to the next step. Protocols with NaOH or OA reduced the concentration of MAP from cattle by 1–2 logs in 4 h, and BAC reduced it by 1 log; HPC did not affect the viability of MAP over a 5-d incubation at concentrations of up to 1% (Jorgensen, 1982; Merkal *et al.*, 1982; Whipple & Merkal, 1983).

Different individuals and combinations of antibiotics have been studied to determine the concentration with maximal effect on contaminating microorganisms, yet with minimal influence on mycobacteria. Antibiotic combinations used include the commercially available PANTA™ Plus (Whittington *et al.*, 1998; Gumber & Whittington, 2007), a mixture of amphotericin B, nalidixic acid, and VAN (Reddacliff *et al.*, 2003; Whittington, 2009), or the less frequently used alone or a combination of penicillin, chloramphenicol, and amphotericin B (Jorgensen, 1982; Whipple *et al.*, 1992). Separately from their inhibitory effect on contaminating bacteria and fungi, these components can also have a negative effect on the growth of mycobacteria, which is strainand antibiotic concentration-dependent (Gumber & Whittington, 2007). Reddacliff *et al.* (2003) reported losses related to carryover effects of the VAN-based incubation (but not statistically significant) when analyzing sheep feces of 1.7 $\log_{10}$, due to 72 h incubation.

When Whipple *et al.* (1992) compared fecal culture techniques on solid media, authors found that conventional sedimentation procedures gave comparable results to those using centrifugation with double incubation and antibiotics (Cornell method) or centrifugation alone. In contrast, Whitlock & Rosenberger (1990) found an increased sensitivity of detection by 3-fold when comparing centrifugation-based to the sedimentation method.

Centrifugal concentration of bovine fecal specimens has been shown to shorten the incubation time required and improve analytical sensitivity (Reddacliff *et al.*, 2003). However, centrifugation did not increase the isolation rate of MAP from fecal specimens when compared to the standard sedimentation method (Kim *et al.*, 1989; Whitlock *et al.*, 1989), and, as for feces, this tends to increase the contamination rate (Reddacliff *et al.*, 2003).

According to Kim *et al.* (1989), double centrifugation-based protocols increase the chance of detecting animals shedding small numbers of MAP organisms. Nevertheless, the same authors reported a significant increase in contamination in centrifuged fecal samples compared to those processed by sedimentation in HEYM cultures (60 vs. 26%), with similar overall detection rates. On the other hand, Ridge (1993) reported a method for bovine fecal sample processing, involving the same protocol (double centrifugation), and then culturing on two stages-culture systems (liquid and solid). The contamination rate was slightly higher for the two-stage method than for HEYM. According to the author, this can be due to the centrifugation process and longer storage of the samples.

Whitlock & Rosenberger (1990) described an alternate centrifugation-double incubation method, with centrifugation speeds lower than the Cornell method, to reduce contamination problems. In their experience, centrifugation increased culture sensitivity by up to three-fold in cattle shedding low numbers of MAP compared with sedimentation. Eamens *et al.* (2000) results reinforce and extend several reports on concentration methods using centrifugation with or without double-incubation found to be more sensitive, compared to those based on sedimentation (from 39 to 68%), since Sweeney *et al.* (1996) found that laboratories employing centrifugation methods had increased detection of positive samples (19%) compared to those using sedimentation as decontamination protocol (15%).

A suggested step-by-step decontamination protocol to be used on bovine fecal samples for the cultivation of MAP in solid media, as defined for the purpose of this review, is presented in Figure 2 and defined according to the results of this SR. To make this decision, we decided to focus on fecal samples only as well as on the lowest contamination rate rather than the MAP recovery, as the latter will depend on other variables that are not directly related to the decontamination process (e.g., MAP viability and quantity in the original sample, elimination intermittency, disease phase, origin of samples, season, and environmental factors).

The centrifugation-one-step HPC seems to be a more reliable protocol for these matrices in terms of the number of samples that have been analyzed with this method (n = 22,154), the lowest contamination rate of the three main protocols presented previously (0.17 vs. 19.9 and

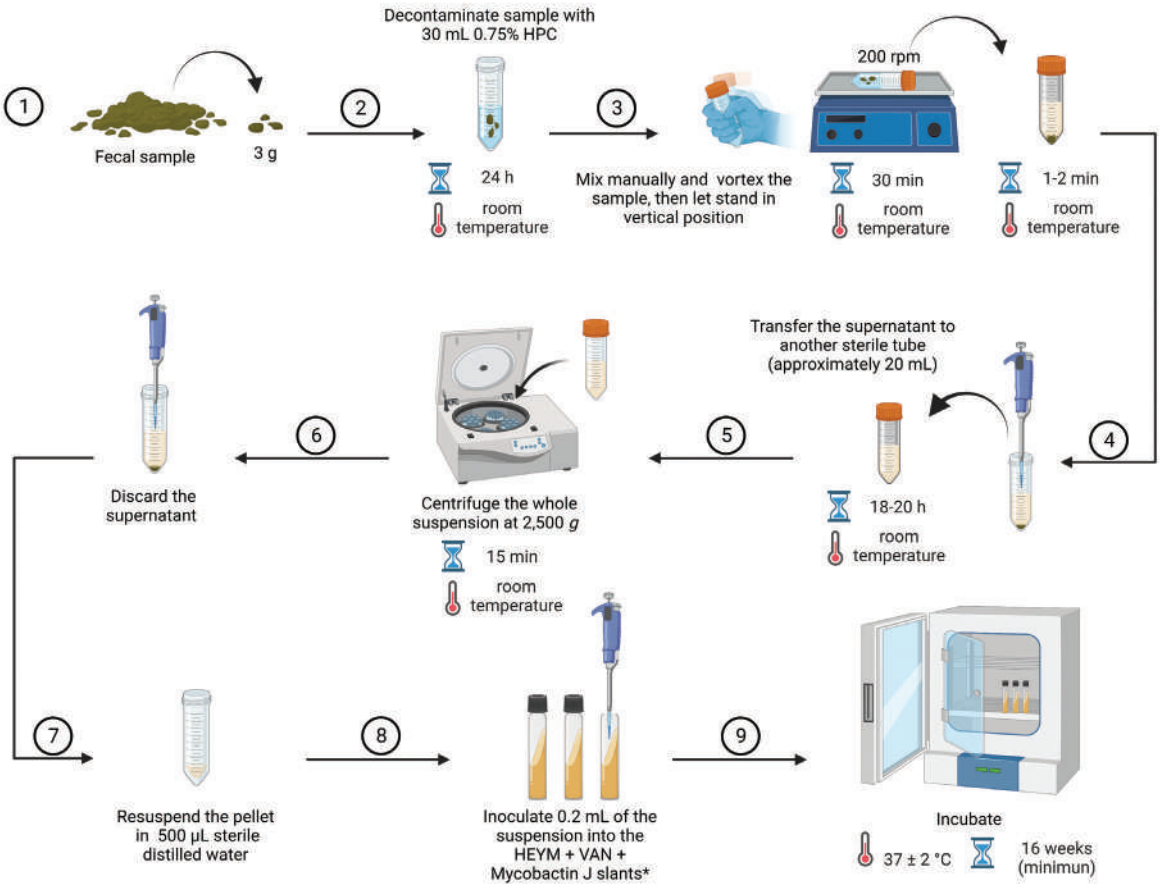

Figure 2.
Suggested decontamination procedure for solid media cultivation of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples (centrifugation-one-step HPC). The steps were defined as follows: 1) sample quantity (g), 2) decontamination solution addition, 3) post-decontamination, 4) incubation, 5) post-incubation centrifugation (g), 6) supernatant handling, 7) pellet handling, 8) inoculation on solid culture media*, and 9) culture incubation.
rpm, revolutions per minute; HEYM, Herrold's Yolk Medium; HPC, hexadecylpyridinium chloride; VAN, amphotericin B, nalidixic acid and vancomycin mixture.
*Commercially available slopes from Becton, Dickinson and Company (HEYM + VAN + Mycobactin J).

17.4%), the "history" of the method (2011-2017), includes the HPC as the chemical decontaminant —recognized as the mainly used decontaminant nowadays, it is supported by the literature as a sensitive protocol in terms of MAP recovery and describes fewer steps to be followed, which translates to less time and instrumentation.

Does this information suggest a change? The answer is, definitively. The consistent use of nonstandard methodologies among studies in this SR makes pooling or comparisons problematic, even given the option of performing a meta-analysis because of the heterogeneity of the data (Dohoo *et al.*, 2014). The difficulty is that there is no true 'gold standard' protocol for MAP in bovine fecal and environmental samples. This problem could be partly addressed by the consistent use of a single reference protocol, thus allowing comparison of diagnostic accuracy across different studies.

A previous SR evaluating rapid tests for bacterial intestinal pathogens in food and feces also reported an overall limited quality of the included studies (Abubakar *et al.*, 2007). Other researchers have concluded that the conduct of SRs and meta-analyses for the evaluation of diagnostic tests have been hampered by the poor quality of reporting of diagnostic studies (Abubakar *et al.*, 2007; Page *et al.*, 2021). Whether similar deficiencies observed in this SR were caused by poor study design or poor reporting is unknown and should be further explored. Efforts have been made in recent years to encourage standardization of methods for reporting primary research and SRs via projects such as the Standards for Reporting Diagnostic Accuracy (STARD) initiative, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, and the development of the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool, all

considered inside the Equators network (https://www.equator-network.org/). Even within the length restrictions imposed by publishing journals, the items listed within these tools must be addressed. Although these tools were developed for application in the human health field, researchers in the areas of veterinary and agri-food public health should be encouraged to adopt similar guidelines. The guidelines provided by these tools should also be considered during the design and conduct of studies on the diagnostic accuracy and related procedures.

The use of a wide array of decontamination protocols, which are compared against an equally wide display of reference protocols, makes it difficult to compare results from different studies. This challenge was highlighted in the current analysis. Results of different studies were found —in some way, contradictory, maybe because of the different matrices (including factors affecting their characteristics, as previously mentioned) and laboratory procedures and facilities. Therefore, there is still a need for further research on the standardization of the protocols, since there seems to be a lack of consistency in the method of isolating MAP from submitted samples at the laboratory level. This void directly affects reports worldwide, leading to incomparable, unrepeatable, and undiscussable results, as culture is still considered the gold standard test for PTB diagnosis (Gilardoni *et al*., 2012; Dane *et al*., 2022).

In addition, it is important to highlight the need for a better definition of the "contamination" outcomes. Contamination by IMs can reduce the diagnostic sensitivity of culture and increase the complexity and cost of confirming the presence of MAP (Whittington, 2009). The detection of mycobacteria other than MAP presents a disadvantage to the routine processing of samples because of the added cost of subculture into additional cultivation and PCR confirmation and a failure to obtain a result for MAP due to contamination. In this same sense, there is a lack of consistency in the MAP culture-related literature about the meaning of "contamination", which makes comparisons between different studies. Contamination can refer to a mixed culture of MAP and other-than MAP microorganisms, a light growth of irrelevant microorganisms or a complete overgrowth of the medium. The lack of case definition for "contamination" when culture outcomes are reported, hinders comparisons among studies.

Then, what other information or research is required on this topic? We suggest separating the research approaches to adapt an optimal decontamination protocol according to each matrix. The above suggested protocol (i.e., centrifugation-one-step HPC), used by four articles in the present SR (Fernández-Silva *et al*., 2011; Donat *et al*., 2015, 2016; Heuvelink *et al*., 2017) could be considered for further analysis since low contamination (almost "negligible") was reported. In addition, contamination-related results should always be reported along with MAP culture results to allow the reader to consider this information while interpreting the results.

Regarding the systematic process carried out per se herein, the authors agreed not to perform the study quality appraisal, as recommended by the PRISMA guidelines, since the answer to the question of interest in this SR is not usually reported as a primary finding. In our case, 27 articles meeting the inclusion criteria were evaluated, and only seven explicitly considered our research question as an objective. The other 20 articles described the decontamination protocol in the Materials and Methods section and reported their results on the contamination rate and MAP recovery in the results or discussion sections. In addition, available quality checklists are not applicable to our purpose, leading to negative results for the internal and external validity appraisal, when in fact they answered our question. Nevertheless, definitive articles incorporated into this SR were always obtained through a consensus on basic eligibility among the authors.

According to the analysis, the concept of contamination was responsible for the limited range of findings. Nevertheless, if we had not considered this, the initial work matrix would have included all the articles that reported solid media, in cattle, and in MAP, which included prevalence and incidence studies, control program evaluation or description, and longitudinal studies, among others, which were out of the purpose of the search. In conclusion, the authors consider the first search to be sufficiently restrictive based on the investigation question.

Our SR has strengths. We followed a written protocol based on a clearly stated and delimited research question. We performed a comprehensive literature search in general-purpose databases, search engines, journals, conference proceedings, book chapters, and books from 1910 to the present using pre-established and explicit inclusion/exclusion criteria. No geographical or temporal constraints were considered in this study. We recognize that our SR contains a modest sample of 27 original articles, given its antiquity in the culture of the microorganism of interest. However, our findings represent the most comprehensive summary of the effects of decontamination protocols on the outcome of solid media for MAP identification.

Our SR had some limitations. When results from snowballing were obtained, we found that the terms "contamina*" and "decontamina*" were the cause that three articles were not detected through the initial searching, being these terms found to be excluded in some specific cases. The grey literature has not been fully considered.

Considering the currently available data, it is difficult to systematically review the literature on this subject, as microbial overgrowth is frequently reported as a secondary outcome instead of a primary objective. The results of this SR demonstrate that there is considerable variability in the percentage of overgrown samples among studies because individual study designs and decontamination protocol characteristics vary considerably among reports.

In conclusion, this SR highlights the need for further refinement of decontamination protocols to minimize the

losses of viable MAP during the processing of bovine fecal and environmental samples because the compilation of information presented herein would orient to protocol improvements and to explore research approaches. We found that sample matrix and quantity, HPC amount, antibiotic concentration, and time-to-contact during incubation, incubation temperature, use of MAP concentration techniques (as filtration), as well as media used and its enrichment type and antibiotic mix, can explain (at some, but no well-established level) the variability in the cultures' outcomes (MAP detection and contamination rate). Finally, it seems that the centrifugation-one-step HPC protocol demonstrates the best results in terms of contamination rate. Nevertheless, it is important to consider the information with the prudence that it deserves and that the outcomes of interest (MAP recovery and contamination rate) may vary from case to case.

Future studies in this area of microbiology should follow standardized guidelines when designing and implementing studies and reporting their results, since the decontamination protocol is a key component in the sensitivity and specificity of the microbiological diagnosis of MAP, which is necessary in the definition of a true global prevalence of MAP.

Competing interests

The authors declare that they have no competing interest.

Author contributions

All the authors contributed to the conception and design of the study. N. M. C. conceived and suggested the design and methods of the SR. The literature search, data analysis, and critical revision of the manuscript were performed by all authors. The first draft of the manuscript was written by NMC, and all the authors commented on the previous versions of the manuscript. Figure 2 was designed by JMH. All authors have read and approved the final manuscript.

Data availability statement

Supplementary data (review protocol, template for data collection forms, and data extracted from included studies) are available upon request to the corresponding author, N.C.V. mariadelp.correa@udea.edu.co

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ORIGINAL ARTICLE

Selenium affects genes associated with immunity and apoptosis in *in vitro* follicles of ewes

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ABSTRACT. Selenium plays an important role in bodily functions. It activates immune cells such as neutrophils and immunity through T cells. However, its role in ovarian follicular gene expression has not been reported. The effects of selenomethionine (SeMet) on gene expression in *in vitro* ewe follicles and their relationship with immunity and apoptosis were studied. Preovulatory follicles were randomly cultured, and SeMet was added to the culture, followed by incubation for 24 h. Total RNA was extracted from follicles and placed on a microarray chip. Gene expression was analyzed using GenArise and classified using the DAVID v6.8 bioinformatics program based on the Gene Ontology and Kyoto Encyclopedia for Genes and Genomes (KEGG). Gene pathways were designed using Cytoscape v3.7.2. There were 2,538 differentially expressed genes (DEGs): 1,228 upregulated and 1,310 downregulated. The upregulated genes were classified into 32, 18, and 12 nodes related to biological processes, cellular components, and molecular functions, respectively. KEGG assigned these genes to 41 metabolic and signaling pathways. 94 genes were involved in eight pathways associated with immune processes. The expression levels of *CD8*, *NFAT2*, and *CD48* were quantified using RT-qPCR. Selenium activated gene expression in the *in vitro* preovulatory follicles of ewes, some of which were related to the immune system. The relationship among *CD8*, *NFAT2*, and *CD48* genes suggests a possible immune pathway related to follicular apoptosis regulation.

Keywords: DNA microarray, preovulatory follicles, organic selenium, apoptosis, follicular immunity.

INTRODUCTION

Designing improved methods for managing production and reproduction is important in animal farms. Therefore, extending the knowledge of how the immune system regulates ovarian events to improve the follicular state is important, because ovarian follicles contain developing oocytes. Consequently, oocytes should be maintained in homeostasis (health) to ensure fertilization and future development of the embryo and fetus, thereby contributing to the enhanced reproductive success of farmed animals.

Selenium is an important trace element in animal physiology. Its main function is to act as an antioxidant by interacting with selenoproteins. It also functions in general immune regulation by promoting T helper cell differentiation. Additionally, it increases the proliferation and development of preovulatory follicles. Selenium increases steroidogenesis in goat granulosa cells. According to Yang *et al.* (2017), Selenium reduced apoptosis (programmed cell death) in rat ovaries and increases embryonic development *in vitro*. Similarly, it also activates gene expression and improves the formation and quality of bovine yolk blastocysts.

In footrot animals, selenium increases the mRNA levels of proteins involved in leukocyte migration. The mRNA expression of selenoproteins and proteins related to lipid metabolism also increased in lambs fed diets containing selenium. Different lipid forms arise during lipid metabolism, including cholesterol, which is a constitutive molecule in the cell membranes and the base of steroid molecules in ovarian follicles.

Several studies have been conducted on ovarian tissues. Ovarian follicle development proceeds through several stages of maturation until differentiation or apoptosis (programmed cell death), which produces high quantities of reactive oxygen species, some of which are linked to immune processes and the maintenance of follicular health. The current study was based on the hypothesis that selenium modifies gene expression in ovarian tissues, some of which may be linked to the immune system. Therefore, this study aimed to investigate the effects of selenomethionine (SeMet) on the follicular tissue of ewes, detect changes in gene expression, and select immunity-related genes with a high probability of being present in the follicles.

MATERIALS AND METHODS

Ovaries of adult ewes of mixed breeds (mainly wool-based) were collected from a local slaughterhouse following Mexican Official Norm NOM-051-ZOO-1995. The ovaries were immersed in cold saline solution (0.9% sodium chloride; 4 °C) with 100 mg L^{-1} gentamicin, kept on ice, and transported to the laboratory.

Subsequently, the adipose tissue and ligaments surrounding the ovaries were removed and the ovaries were repeatedly washed with cold saline solution (habitual handling). Follicles were extracted from the rest of the ovar-

ian tissue under a stereoscopic microscope (Carl Zeiss de México S.A. de C. V., CDMX, Mexico) and follicular diameter was measured using a caliper rule. Preovulatory follicles (diameter ≥ 6 mm) were dissected from ovaries under sterile conditions using a scalpel and dissecting forceps. Before *in vitro* culture, the follicles were immersed in a 2:1 mixture of culture medium (MEM; Sigma-Aldrich, St. Louis, MO, S.A de RL. de CV., Toluca, Estado de México) and saline solution with 100 mg L-1 gentamicin.

In vitro culture

The experimental design for the culture included two treatments: a) without SeMet (control) and b) with SeMet (18 replicates per treatment).

Thirty-six preovulatory follicles were selected and placed in Eppendorf tubes containing 1 mL culture medium. The control group consisted of 18 preovulatory follicles in culture medium containing 100 IU mL^{-1} equine chorionic gonadotropin (eCG; VIRBAC XICO S.A. de C.V.). The treatment consisted of 18 follicles (six tubes with three follicles each) treated with 10 ng mL^{-1} SeMet (Sigma-Aldrich Química, S.A de RL. de CV. Toluca, Estado de México) in addition to other components of the control. The follicles were incubated for 24 h; incubations were carried out at 37°C in 95% humidified air with 5% CO^2. After 24 h, the follicles were removed from the culture medium, placed in clean Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C until total RNA extraction.

Total RNA extraction

Total RNA was extracted from follicles using TRIzol Reagent and Invitrogen (Thermo Fisher Scientific Inc. MA, USA) according to the manufacturer's instructions. Total RNA was quantitatively and qualitatively evaluated (based on absorbance) using a NanoDrop spectrophotometer (ND-2000; Thermo Scientific, MA, USA), and band separation was performed on 1.5% agarose gel (Green & Sambrook, 2012). Total RNA samples were used for cDNA synthesis using SuperScript IV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific Inc., MA, USA), and microarray hybridization (Vallée *et al.*, 2006) was performed using the cDNA microarray M22K _11_06. Alexa 555 and Alexa 647 were used to label samples developed in the DNA Microarray Unit of the National Autonomous University of Mexico.

Evaluation of genes related to immunity and apoptosis in *in vitro* SeMet-treated follicles

The three differentially expressed genes (DEGs) that were overexpressed in *in vitro* SeMet-treated follicles were selected and validated by RT-qPCR. Furthermore, differences in expression among the three genes were analyzed using primers (Table 1) based on the ewe transcripts (the primers were designed using the CDS of ewe genes reported in GenBank and Primer3 software) instead of the expression determined using mouse-based microarrays. RT-qPCR was performed at the Genomic Services Unit (LANGEBIO-CINVESTAV, Gto, Mexico) using three technical replicates. The ACTB (beta-actin) gene was used as a housekeeping gene. The $2^{-\Delta\Delta CT}$ quantification method was used for qPCR.

Quantification and classification of data

To quantify and analyze gene expression, the fluorescence data emitted by the microarray were normalized us-

Table 1.
Primers used for qPCR of the three upregulated genes involved in immunity and apoptotic process in *in vitro* SeMet-treated follicles of ewes.

Target gene	Primer sequence (5´-3´)	Sense	Product size (bp)	GenBank reference
ACTB	CATCGGCAATGAGCGGTTCC	Forward	146	NM_001009784
	CCGTGTTGGCGTAGAGGT	Reverse		
CD8A	GGAGTGAACTGAACCCTGGA	Forward	225	XM_027966754
	TGACCCAGGAGCATGTTTGA	Reverse		
NFATc2	CCACTTTTCTCCAACAGCCC	Forward	193	XM_027977331
	GAACCCACCCACTGAAACAC	Reverse		
CD48	GCTTGGCATCCTTCTCATGG	Forward	250	XM_012183742
	GCCCTTCTCCGAGTCTTTCT	Reverse		

ing the GenArise microarray analysis tool, and genes with z-scores between 1.5 and 2 were considered DEGs.

The DEGs were further classified based on term annotation using the Gene Ontology (GO) database, which considers $p \leq 0.01$ and $p < 0.05$, and the Kyoto Encyclopedia for Genes and Genomes (KEGG) database of metabolic pathways and signaling ($p < 0.05$) using DAVID Bioinformatics Resources v6.8. The interactions and pathways (gene networks) of genes with immune activity were studied using Cytoscape v3.7.2.

RESULTS

After comparing fluorescence emission between follicles exposed to selenium and those not exposed to selenium using mouse microarray data, 2,538 DEGs were identified. Of these, 1,228 and 1,310 genes were upregulated and downregulated, respectively.

GO and KEGG classification of DEGs in *in vitro* SeMet-treated preovulatory follicles

A total of 1,411 genes were analyzed using the DAVID program for GO analysis and categorized into three functional nodes ($p < 0.01$): 32 nodes related to biological processes (BP), 18 related to cellular components (CC), and 12 related to molecular functions (MF). According to GO analysis, most genes were ranked in the BP category. The upregulated genes under BP were related to the transcription, transport, and development of multicellular organisms, whereas those under CC included genes encoding proteins related to the cytoplasm, nucleus, cytosol, and membrane. (Figure 1).

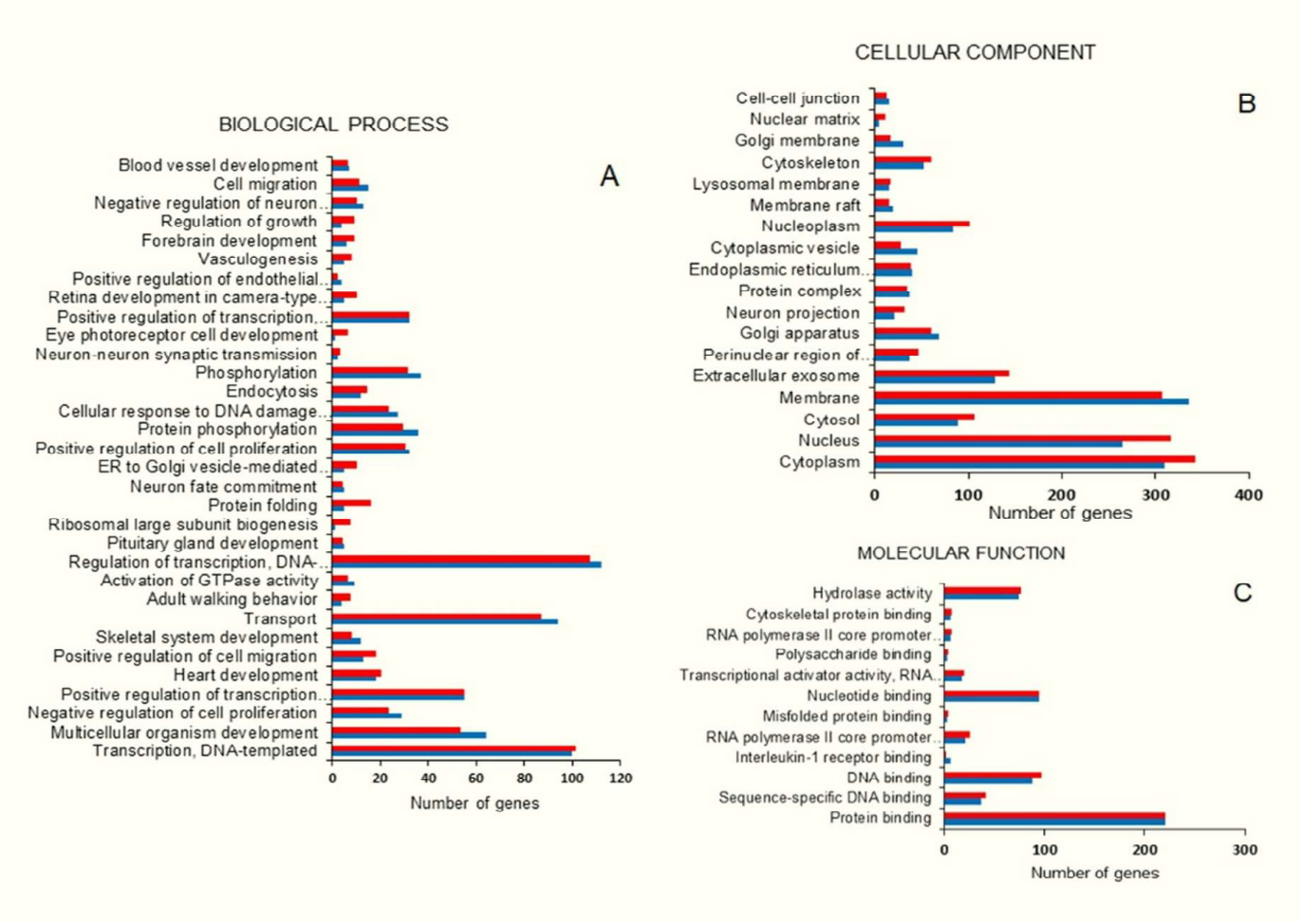

Figure 1.
Gene Ontology (GO)-based number and ranking of differentially expressed genes in *in vitro* SeMet-treated preovulatory follicles of ewes ($p \leq 0.01$). Red bars = upregulated genes; blue bars = downregulated genes. A: Genes involved in biological processes; B: Genes involved in cellular components; C: Genes involved in molecular functions.

One hundred ninety-five DEGs were associated with immune processes, and GO analysis classified them into 115 functional genes in BP, 42 in CC, and 38 in MF ($p < 0.05$). Most of the upregulated genes were related to positive (40) and negative (59) regulation of apoptosis (Table 2; $p < 0.01$).

DEGs related to metabolic and signaling pathways were classified into 41 pathways using KEGG ($p \leq 0.05$). The pathways with the highest number of upregulated genes were related to cancer, infectious diseases, and activation and signaling (e.g., mitogen-activated protein kinase

Table 2.
Genes associated with biological processes, specifically the immune system, in *in vitro* SeMet-treated preovulatory ovarian follicles of ewes.

Term: GO:0006897 ~endocytosis. Fold enrichment: 1.8, p = 0.0048	**Up-regulated** (14): *CD209B*, *RAB1A*, *ARHGAP27*, *AP2M1*, *APP*, *ARR3*, *EPS15*, *PIK3CB*, *PSTPIP1*, *PACSIN1*, *PACSIN3*, *SORT1*, *SNX1*, *SNX4*. **Down-regulated** (12): *ATP9A*, *CD209B*, *GAPVD1*, *HRAS*, *RAB34*, *ARHGAP27*, *WIPF2*, *ARC*, *AP2M1*, *APP*, *ARR3*, *A4*.
Term: GO:2000353 ~positive regulation of endothelial cells apoptotic process. Fold enrichment: 4.7, p = 0.0066	**Up-regulated** (2): *FOXO3*, *AGER*. **Down-regulated** (4): *AKR1C18*, *COL18A1*, *PRKCI*, *RGCC*.
Term: GO:0043524 ~negative regulation of neuron apoptotic process. Fold enrichment: 1.80, p =0.0066	**Up-regulated** (10): *ISL1*, *JAK2*, *KRAS*, *CACNA1A*, *MTNR1B*, *MT1*, *NGF*, *NRBP2*, *SOD1*, *UBE2V2*. **Down-regulated** (13): *BTG2*, *HRAS*, *CORO1A*, *EN1*, *FOXB1*, *FZD9*, *HSPD1*, *HIPK2*, *LGMN*, *MDK*, *NPM1*, *PRKCI*, *SIX4*.
Term: GO:0043065 ~positive regulation of apoptotic process. Fold enrichment: 1.50, p = 0.0108	**Up-regulated** (18): *JAK2*, *SOX4*, *B4GALT1*, *WT1*, *AIFM1*, *AGER*, *CASP1*, *CTNNB1*, *CCAR1*, *DAPK3*, *EEF1E1*, *FOXO3*, *MSX2*, *NGF*, *NET1*, *PTEN*, *ZAK*, *TOP2A*. **Down-regulated** (21): *CLIP3*, *RBCK1*, *APBB2*, *CTNNBL1*, *CLU*, *DHODH*, *HSPD1*, *HMGA2*, *ING5*, *IP6K2*, *IL24*, *MUC2*, *LPAR1*, *NTRK3*, *KCNMA1*, *SFRP1*, *SAV1*, *STK4*, *TEX261*, *TFAP4*, *TGFB1*.
Term: GO:0010667 ~negative regulation of cardiac muscle cells apoptotic process. Fold enrichment: 3.38, p = 0.0145	**Up-regulated** (5): *PCMT1*, *QK*, *JAK2*, *HSF1*, *NFE2L2*. **Down-regulated** (2): *GHRH*, *NPM1*.
Term: GO:0006909 ~phagocytosis. Fold enrichment: 2.41, p = 0.0205	**Up-regulated** (4): *ANXA3*, *MEGF10*, *TULP1*, *TUSC2*. **Down-regulated** (6): *GATA2*, *CORO1A*, *EIF2AK1*, *HCK*, *ABL2*, *VAV1*.
Term: GO:0043066 ~negative regulation of apoptotic process. Fold enrichment: 1.31, p = 0.0286	**Up-regulated** (32): *ARF4*, *ARAF*, *BCL11B*, *CD38*, *CD74*, *HHIP*, *JAK2*, *ARHGAP10*, *SMARCA4*, *TRIAP1*, *WT1*, *AIPL1*, *AVEN*, *CRYAB*, *CBS*, *TEK*, *EPCAM*, *FXN*, *GAS1*, *MAEA*, *MSX2*, *PROP1*, *PRDX5*, *PTEN*, *PLAC8*, *PLK1*, *RHBDD1*, *SLC40A1*, *SPHK1*, *SOD1*, *TEX11*, *ZFP830*. **Down-regulated** (27): *OGG1*, *BTG2*, *LIMS1*, *PTK2*, *STIL*, *ALB*, *AKR1B3*, *APBB2*, *ARNT2*, *BFAR*, *CLU*, *DPEP1*, *GNAQ*, *HSPD1*, *HELLS*, *HCK*, *HMGA2*, *IL24*, *LTK*, *NPM1*, *PAX4*, *KCNJ1*, *PRKAA2*, *SFRP1*, *SIX4*, *SPHK2*, *UCP2*.
Term: GO:0038096 ~gamma receptor signaling pathway involved in phagocytosis. Fold enrichment: 9.41, p = 0.0341	**Up-regulated**: 0 **Up-regulated** (3): *WAS*, *WASL*, *CDC42*.
Term: GO:0042110 ~T-cells activation. Fold enrichment: 2.58, p = 0.0495	**Up-regulated** (2): *CD48*, *CD8A*. **Down-regulated** (5): *WAS*, *HSPD1*, *ITGAV*, *TGFB1*, *VAV1*.
Term: GO:0030097 ~hemopoiesis. Fold enrichment: 1.90, p = 0.0395	**Up-regulated** (6): *CD34*, *TIPARP*, *ADD2*, *CTNNB1*, *TEK*, *GFI1*. **Down-regulated** (7): *GATA2*, *BRCA2*, *CUL4A*, *KIRREL3*, *PICALM*, *RUNX1*, *SFRP1*.

(MAPK) and Ca^{2+} signaling). Eight gene nodes were related to immunity: natural killer cell-mediated cytotoxicity, Wnt signaling, B-cell receptor signaling, T-cell receptor signaling, transendothelial migration of leukocytes, MAPK signaling, inflammatory mediator regulation of transient receptor potential (TRP) channels, and phagocytosis mediated by crystallizable fragment (Fc) gamma R (Figure 2).

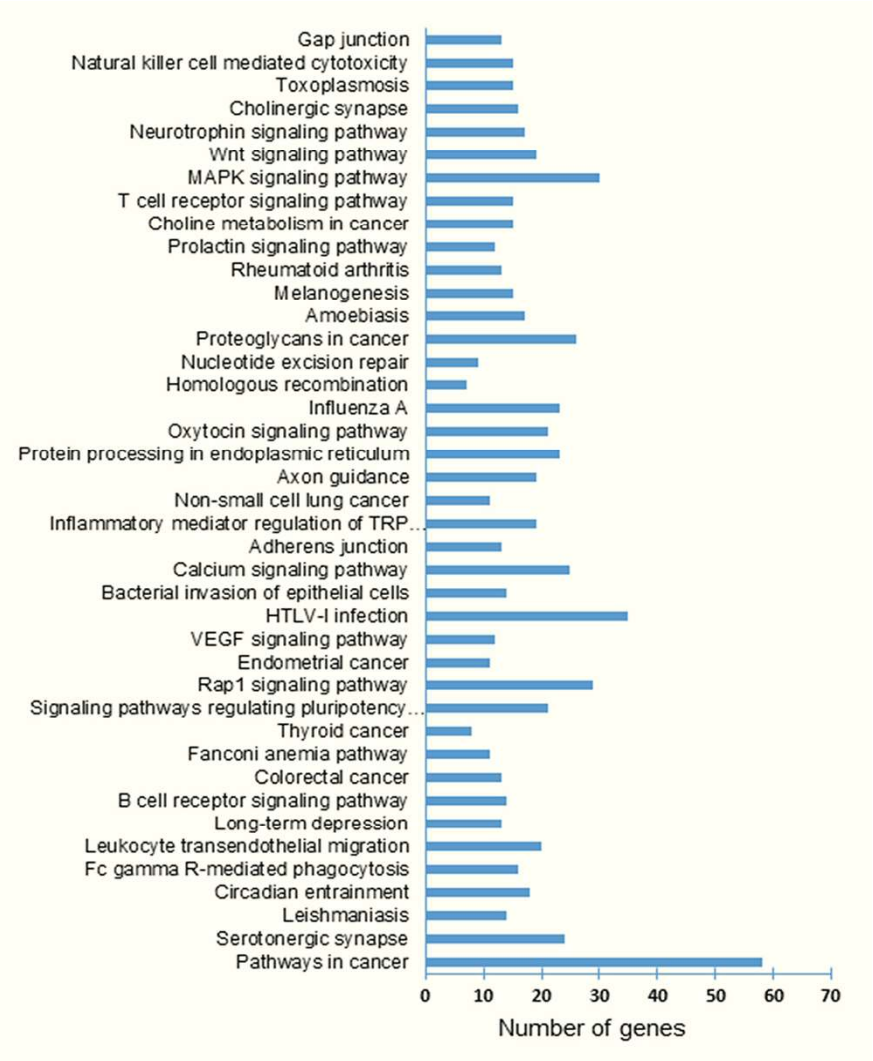

Figure 2.
Kyoto Encyclopedia for Genes and Genomes (KEGG)-based classification of differentially expressed genes (DEGs) related to metabolic and signaling pathways in *in vitro* SeMet-treated preovulatory follicles of ewes.

Immune gene network in *in vitro* SeMet-treated preovulatory follicles of ewes

The gene expression network involved in the immune system of *in vitro* SeMet-treated preovulatory follicles of ewes was constructed using 100 upregulated and downregulated genes.

Using GO terms, eight gene nodes were found to be related to cellular and humoral immunity: T-cell receptor signaling, B-cell receptor signaling, natural killer cell-mediated cytotoxicity, and leukocyte transendothelial migration. The upregulated genes included *CACNA1*, *VAV2*, *PIK3CD*, and *WNT8A*. *CD48* is linked to the natural killer cell pathway and mediates cytotoxicity, which, in turn, is linked to the T-cell receptor signaling pathway mediated by *NFATC2* to communicate with *CD8A*. The pathways used by *CD48*, *NFATC2*, and *CD8A* are active immune pathways involved in cell apoptosis (Figure 3; lines with stars). *NFATC2* was not only associated with *CD48* and *CD8A* through the natural killer cell-mediated cytotoxicity pathway and T cell receptor signaling pathway, but was also related to the *WNT* signal-

ing pathway and B cell receptor signaling pathway (Figure 3; lines with squares); the latter involved humoral immunity. The *CD8A*, *NFATC2*, and *CD48* genes were also located in these pathways and subsequently validated by RT-qPCR.

RT-qPCR validation of the three genes involved in immunity and apoptosis in *in vitro* SeMet-treated preovulatory follicles of ewes

Changes in *CD8A*, *CD48*, and *NFATC2* gene expression in *in vitro* SeMet-treated preovulatory follicles of ewes observed using a mouse gene-based microarray were further confirmed using RT-qPCR, and gene expression was compared between the two techniques (Figure 4). The gene expression signals obtained using microarrays were higher than those obtained using RT-qPCR.

DISCUSSION

In this study, 2,538 DEGs were identified, suggesting that *in vitro* selenium (10 ng mL^{-1}) treatment of preovulatory follicles affects the transcriptome. These results are in accordance with those observed in ewes supplemented with a high concentration of organic selenium (0.40 mg), wherein 1,186 differentially expressed transcripts, includ-

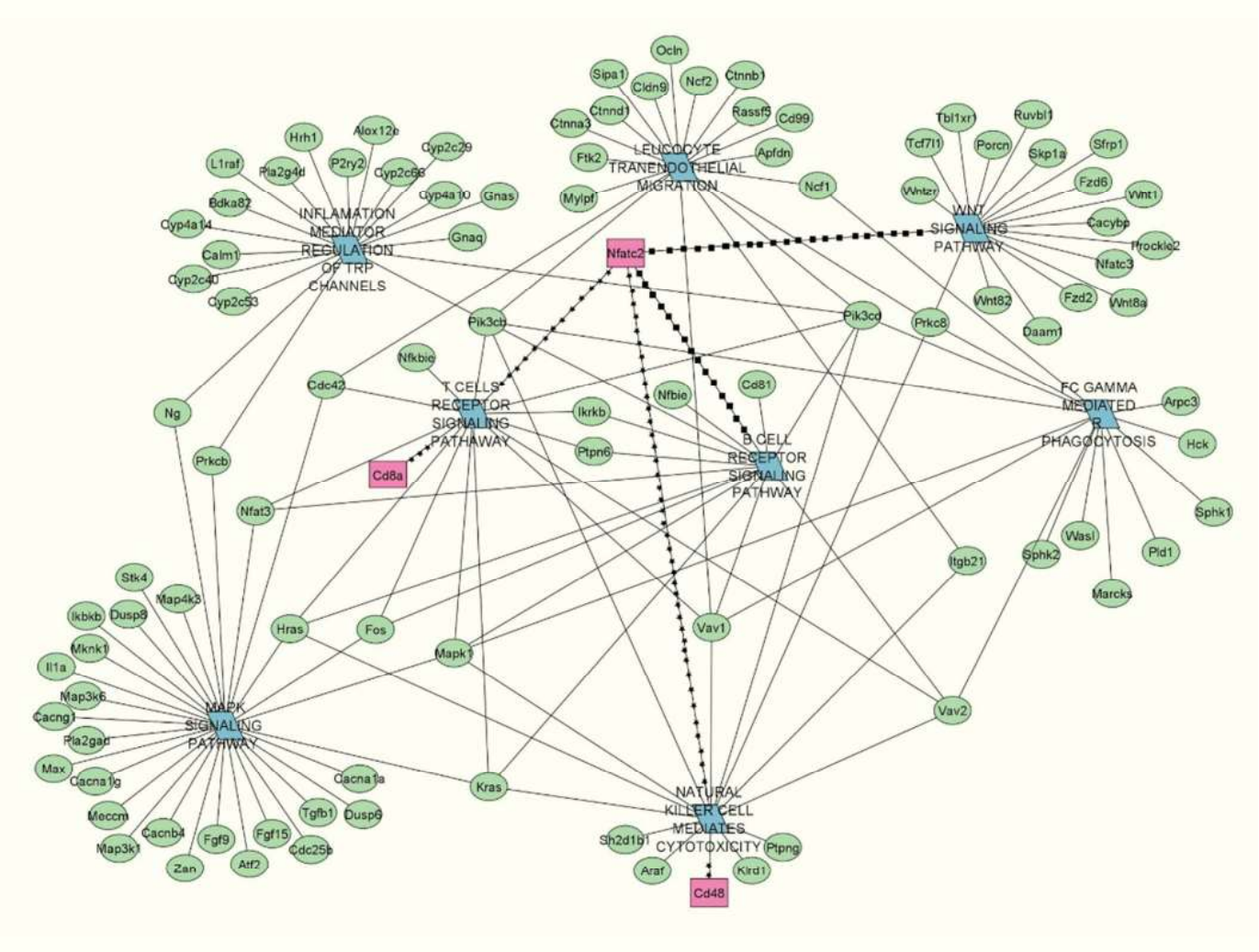

Figure 3.
Network of genes involved in the immune process of *in vitro* SeMet-treated preovulatory follicles of ewes. Circles represent 105 genes: blue polygon, principal family nodes; pink rectangles and dotted lines; genes tested by RT-qPCR and involved in the apoptotic process by two family nodes related to immunity.

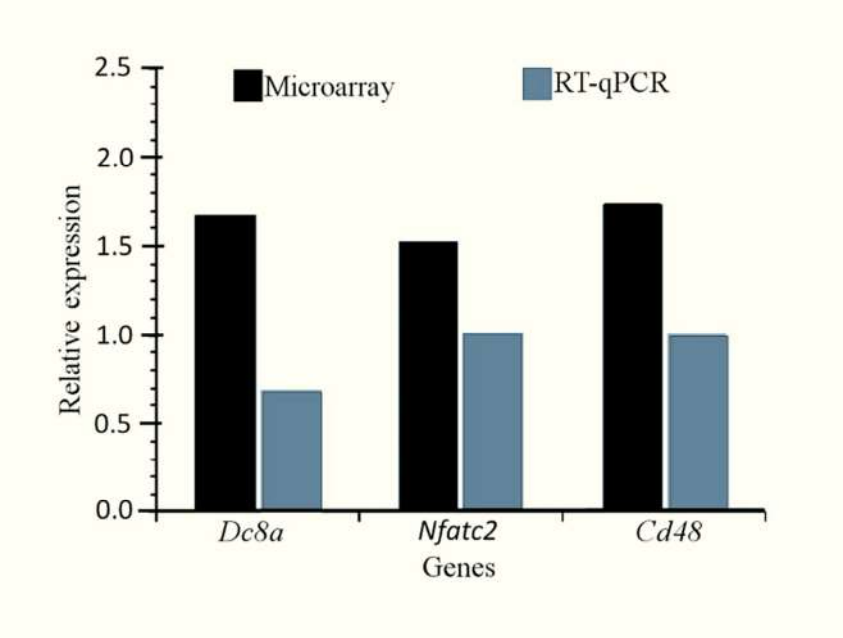

Figure 4.
The three immunity-related genes expressed in *in vitro* SeMet-treated preovulatory follicles of ewes and validated using RT-qPCR.

ing those of genes related to immunity, were observed. However, the study by Elgendy *et al.* (2016) was conducted on blood cells and not on ovarian follicles, as in the present study. Notably, mouse microarray results were consistent with the genes expressed in ovine follicles. Furthermore, Elgendy *et al.* (2016) reported that the number of transcripts detected using a mouse microarray was higher than that detected using an ovine microarray.

According to GO, most DEGs were classified under the BP category, specifically those related to transcription processes, which can be attributed to the activation of selenoprotein transcription and elevation of biological activities involving selenium.

In this study, functional analysis of these genes identified 41 pathways, including those related to the immune system. These results are similar to those reported by Song *et al.* (2013) for pig leukocytes treated with selenium (0.54 mg kg^{-1} of diet), wherein 28 upregulated and 24 downregulated genes were observed. The first factor is related to immunity. Similar results have been reported by Elgendy *et al.* (2016). Collectively, these results highlight the importance of selenium in the genetic regulation of immune processes in various tissues.

Functional clusters, particularly those related to cell activity and death (apoptosis), were identified in 195 genes. These results can be attributed to the follicular development process in which both events (i.e., cell differentiation and death) occur in the follicles.

Construction of a gene network with 94 genes highlighted the nodes and pathways related to immune processes. Among these nodes, there are gene interactions for apoptosis process regulation, namely, a signaling node of the T cell receptor that, together with interleukins, acts in many immunological conditions, and a node of cytotoxicity mediated by natural killer cells that are related to multiple functions in the immune process and during apoptosis. This study verifies the direct relationship between these cell nodes.

These results indicate that the participation of these gene nodes, together with leukocyte transendothelial migration, is required to regulate preovulatory follicle activity and apoptosis. Previously, it has been observed that selenium causes autophagy and apoptosis in cancer cells. Furthermore, it plays an alternative role in apoptosis owing to its antioxidant capacity and free radical scavenging activity. Therefore, at the follicular level, while some cells are in the process of apoptosis, others overcome the event and move towards differentiation, which can also be verified by the sub-regulation of genes involved in follicular cell proliferation observed in this study. Considering the differences in follicular cell populations, the effects of Se on gene expression in different follicular cell populations should be studied further.

Three genes involved in immunity, *CD8A*, *NFATC2*, and *CD48*, were evaluated by RT-qPCR. These genes were upregulated in preovulatory follicles in response to SeMet.

CD8 and *CD4* appear to be involved in signaling damage during follicular apoptosis. The *CD8A* and *CD4* genes are essential because their protein products act as receptors for the major histocompatibility complex (MHC), which facilitates the presentation of antigens to T cells and activates specific immune responses. CD4(+) T cells are essential for the formation of protective memory CD8(+) T cells following infection or immunization. Therefore, CD8 is important for maintaining immunity throughout the lifetime, which can act during repeated attacks by microorganisms or against cyclic physiological events, such as apoptosis, during which it is necessary to eliminate toxic products from the cell.

NFATC2 encodes a transcription factor that regulates the genes and proteins involved in immune responses. For example, *NFATC1* and *NFATC2* deficient T cells cause a scarcity of Th cytokine production, hyperactivation of B cells, and *NFAT* controls the exhaustion of CD8+ T cells. Therefore, in the absence of *NFAT*, T cells exhibit decreased immune protection. The relationship between *NFATC2* and *CD8* was observed in our study, and *CD8*, *NFATC2*, and *CD48* were found to be directly linked. Another important effect of *NFAT* is cytokine regulation, which plays an important role in regulating apoptosis and follicular atresia. Additionally, NFATC2 a member of the *NFAT* family, is a transcription factor that activates the expression of cytokine and T cell genes and is involved in the induction and apoptosis of T lymphocytes.

Similar to *CD8* and *NFATC2*, *CD48* is linked to the natural killer cell-mediated cytotoxicity pathway. *CD48* activates innate lymphoid cell progenitors in humans. This receptor is present in different cell types, including natural killer cells and innate lymphocytes. *CD48* also participates in immune-cell adhesion. Similarly, *CD48* on T cells can promote T cell receptor signaling and activation and anchorage between GPI or Lck. *CD48* allows them to contact cholesterol-rich lipid rafts, facilitating intracellular calcium flux in a cholesterol-dependent mechanism. Both follicle and luteal tissues have large amounts of lipids (cholesterol) for steroidogenesis, which may explain why the expression of this gene was observed in this study. The results of this study are related to the observations of Basini & Tamanini (2000), who reported an increase in steroidogenic activity of granulosa cells following selenium administration.

Selenium acts as an immunostimulator by activating T cell proliferation and natural killer cell differentiation, and by stimulating other innate immune cells. To our knowledge, this is the first study to determine the effects of Se on gene expression in preovulatory follicles *in vitro*. Furthermore, the pathway through which *CD8A*, *NFATC2*, and *CD48* were quantitatively (RT-qPCR) validated is related to the immune response triggered by cytotoxicity. Based on the pathway formed by *CD8A*, *NFATC2*, and *CD48*, we suggest that these genes could be part of the immune control during apoptosis. Another reason may be that the differentiation process of follicular cells is similar to that observed in T cells and natural killer cells. The results of this study highlight the potential importance of selenium in enhancing immunity in the preovulatory follicles.

The *CD8A*, *NFATC2*, and *CD48* genes were validated using RT-qPCR. Although the gene expression levels differed between the two techniques, they were complementary, implying that one technique was better than the other owing to the precision of each technique, sequence, selection of primers, primer design, and other experimental conditions. Regardless of the signals detected, the genes identified using mouse microarrays were also detected using RT-qPCR based on ewe genes. Independent of the technique used, the expression of *CD8A*, *NFATC2*, and *CD48* in *in vitro* SeMet-treated preovulatory follicles of ewes was verified. Remarkably, a microarray based on mouse gene libraries was helpful in the absence of an ovine-origin microarray. Verification of the expression of *CD8A*, *NFATC2*, and *CD48* could have been carried out using other techniques in addition to qPCR, which may be a limitation of our study. Finally, this study provides useful information regarding the reproductive system of ewes. Selenium administration can improve reproduction in ewes by increasing the quality of the follicular immune system and the possibility of mature oocytes.

CONCLUSIONS

SeMet activates several genes, including those related to the immune system, in the preovulatory follicles of ewes *in vitro*, some of which are likely involved in follicular cell apoptosis. If follicular apoptosis is diminished, free radical levels and follicular toxicity are decreased. This cascade of events can increase follicular development and number of lambs per reproductive season.

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Conflict of Interest

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ORIGINAL ARTICLE

Carvacrol-loaded invasomes biocidal effect against multidrug resistant isolates of Enterobacteriaceae and housefly

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ABSTRACT. The current study aimed to evaluate the antimicrobial activity of carvacrol-loaded invasomes (CLI) against multidrug-resistant (MDR) Enterobacteriaceae and its mechanical vector, the housefly. CLI were prepared and characterized in the laboratory. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* enterica subsp. *enterica* serovar Enteritidis, Salmonella *enterica* subsp. *enterica* serovar Typhimurium, and *Klebsiella oxytoca* were among the MDR enterobacteriaceae stains investigated. These strains were first isolated and identified from naturally infected chickens. The antibacterial activity of CLI against the MDR isolates was evaluated using the diffusion method. In addition, the insecticidal activity of CLI against housefly larvae and pupae was tested. The MDR index of all evaluated isolates was greater than 20%, indicating that they were all multidrug-resistant. CLI decreased the growth of all isolates except *S. Typhimurium* and *P. aeruginosa* at a dose of 0.0125%; however, pure carvacrol inhibited the growth of only *Klebsiella* oxytoca. Furthermore, both CLI and pure carvacrol inhibited *Klebsiella oxytoca* growth at different concentrations. CLI inhibited *E. coli* and *S. enteritidis* at lower concentrations than pure carvacrol, even at a doubled concentration. Carvacrol and CLI caused significant larval mortality even at low concentrations, with LC50 reached at concentartions of 2.54 and 2.19 µl/ml, respectively. Furthermore, at a low concentration of 3.125 µl/ml, both elicited a high percentage inhibition rate (PIR) in pupae. In conclusion, CLI demonstrated substantial antibacterial action, particularly against MDR isolates, as well as pesticide activity against houseflies.

Keywords: carvacrol, invasomes, drug resistance, antimicrobial, *Musca domestica*.

INTRODUCTION

Antimicrobial drugs are frequently given to chickens to promote growth and prevent diseases, but prolonged usage or improper use commonly results in bacterial resistance (Fielding *et al*., 2012), a problem that is becoming increasingly prevalent in humans and animals worldwide (Spellberg, 2014). The WHO proposed global programs for both human and animal surveillance in light of this increasing threat. Antimicrobial resistance in chickens is a common problem in developing countries because of the indiscriminate use of antibiotics as feed additives and the prophylactic treatment of infectious diseases. Because there are few or no alternative effective antimicrobial drugs available for the treatment of diseases caused by these bacteria, the evolution of bacterial resistance to antimicrobial treatments has become a serious public health concern (Al Azad *et al*., 2019). Consequently, the chicken industry has recently piqued interest in investigating phytobiotics as an alternative to synthetic antibiotics (Altay *et al*., 2022).

Escherichia coli is a bacterium that has a special place in the microbiological community because it not only causes serious infections in humans and animals but also contributes significantly to the autochthonous microbiota of different species. The potential transmission of virulent and/or resistant *E. coli* from animals to humans via several pathways, including direct contact, contact with animal excretions, and the food chain, is a major source of concern. Additionally, *E. coli* is a significant reservoir of resistance genes, which could be the reason why some treatments in both human and veterinary medicine are ineffective (Agatha *et al*., 2023; Bassi *et al*., 2023). Multidrug resistance in *E. coli* has recently become a global concern (Poirel *et al*., 2018).

Musca domestica, the housefly, is a vector for over 100 human and animal diseases, mainly food-borne pathogens (Kumar *et al*., 2013, 2014). Chemical insecticides are often

used to control houseflies. Long-term use of these chemical insecticides has serious consequences for human and animal health as well as detrimental effects on the environment (Kumar *et al.*, 2012). Given the previously noted increased rate of antibiotic resistance as well as the side effects and disadvantages of insecticides, novel approaches to overcome these obstacles are urgently required. As a result, there is an urgent need to investigate better alternatives to antibiotics to ensure the production of safe and profitable poultry.

Natural bioactive compounds derived from plants could be potential alternative candidates (Pavela, 2013; Abdel-Baki *et al.*, 2021). One of these compounds is carvacrol, a phenolic monoterpenoid found in essential oils of different plant species. Carvacrol has been demonstrated to have several biological effects, including antibacterial and antifungal properties (Chavan & Tupe, 2014), antiviral activity (Sánchez *et al.*, 2015), antioxidant properties, immune response regulation (Khazdair *et al.*, 2018), and anti-inflammatory properties (Fitsiou *et al.*, 2016).

According to Di Pasqua *et al.* (2010), carvacrol interacts with the cell membrane via hydrogen bonding, making the membranes and mitochondria more permeable and disintegrating the outer cell membrane. Several *in vitro* studies have revealed that carvacrol and thymol have potent antibacterial activity against pathogenic bacteria, including *E. coli* and *S.* typhimurium (Gholami-Ahangaran *et al.*, 2020). Carvacrol is more effective against gram-positive bacteria than gram-negative bacteria; it damages bacterial membranes, reduces ATP generation, and consequently energy-dependent cell functions (Nostro & Papalia, 2012). However, the application of carvacrol is limited by its high volatility, low water solubility, and low stability (Donsì *et al.*, 2014; Locci *et al.*, 2004).

Carvacrol nanoformulations, such as nanocarriers, are a strategy to overcome these constraints. Nanocarriers have been utilized to increase the chemical and physical stability of essential oils, reduce organoleptic alterations, and promote biological activity (Moraes-Lovison *et al.*, 2017; Ryu *et al.*, 2018; Noori *et al.*, 2018; Chuesiang *et al.*, 2019). Invasomes are one of these nanocarriers that have been used frequently (Kamran *et al.*, 2016). Invasomes are composed of unsaturated phospholipids, water, and trace amounts of ethanol and terpenes, which increase permeability and bioavailability (Aslam *et al.*, 2015; Dwivedi *et al.*, 2016; Kumar *et al.*, 2022).

In the current study, a carvacrol-loaded invasome (CLI) was similarly prepared, with terpenes responsible for the biocidal effect enhancing CLI solubility and penetration of the larval cuticle by disrupting lipid/protein layers and/or removing skin micro-ingredients required for skin barrier maintenance (Sapra *et al.*, 2008; Kumar *et al.*, 2022).

In the present study, the prepared carvacrol-loaded invasome (CLI) was tested against multidrug resistant isolates of *E. coli*, *S. enterica* Enteritidis, *S. enterica* Typhimurium, and *P. aeruginosa*, as well as the mechanical vector of these microorganisms' housefly.

MATERIALS AND METHODS

Preparation and characterization of Carvacrol-Loaded Invasome (CLI)

In our laboratory, a carvacrol-loaded invasome (CLI) was prepared and characterized as described by Gamal *et al.* (2023). In brief, a carvacrol-loaded invasome (CLI) formulation was produced using a thin hydration method. Carvacrol (10 mg), cineole (1% v/v), cholesterol (0.15 %w/w), and phospholipid (3% w/w) were dissolved in 10 mL organic solution of chloroform and methanol (3:1). This solution was evaporated under vacuum using a Stuart rotary evaporator (RE300, UK) at a speed of 100 rpm and temperature of 40 °C. During evaporation, a thin layer of invasomes formed inside the flask. At 40 °C for an hour, isotonic phosphate buffer (IPB, pH 5.5) solution and ethanol (3% v/v) solution were added to hydrate the lipid film. The carvacrol-loaded invasome (CLI) formulation was developed, sonicated, and kept at 4 °C.

Bacterial isolates used in the study

Five types of gram-negative bacteria, including *E. coli*, *P. aeruginosa*, *S.* Enteritidis, *S.* Typhimurium, and *Klebsiella oxytoca*, were found in naturally infected chicken. These chicken isolates affected 10,000 birds aged 1-21 days and were collected from ten broiler flocks between 2020 and 2021. The samples were examined for the presence of pathogenic bacteria that cause conjunctivitis, arthritis, enteritis, and diarrhea. The isolates were identified and serotyped according to Quinn *et al.* (2011). Stock cultures of these bacteria were grown on Mueller-Hinton agar at 37 °C for 24 h for subsequent bioassays. Antimicrobial susceptibility testing and determination of multi-drug resistance index (MDRI).

All bacterial isolates were tested for sensitivity to 12 different antimicrobial agents using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (Oxoid Ltd., Basingstoke, UK), according to the CLSI guidelines (2018). Resistance to three or more antibiotics from different groups represents multidrug resistance (MDR). Individual isolates' MDR index (MDRI) was calculated by dividing the number of antimicrobials to which the isolate was resistant by the total number of antibiotics to which the isolate had been exposed (Chandran *et al.*, 2008). Isolates with MDRI values greater than 0.2 or 20% were considered highly resistant.

$$\text{MDR index} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

Determination of the effect of CV and CLI on bacterial pathogen growth using the agar diffusion method. Different concentrations of pure carvacrol and CLI (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 µl/ml) were prepared by diluting with 1% DMSO. The antibacterial activity of the prepared concentrations was tested against MDR isolates as described by Jeff-Agboola *et al.* (2012). Briefly, bacteria were cultivated on tryptone soya agar at 37 °C for 24 h before being suspended in physiological saline (0.9% NaCl) and adjusted to 0.5×108 CFU. Muller-Hinton agar was prepared and autoclaved at 121 °C for 15 min. before being maintained at 55 °C. The tested oils were then combined with TSA according to the tested concentrations. The oil-agar medium (10 ml) was then solidified in sterilized Petri dishes. Equal volumes of bacterial suspensions were inoculated and spread on agar plates. The plates were then incubated at 37 °C for 24-48h. The cells were examined for bacterial colony growth inhibition.

Housefly

Adult houseflies captured in the field using a sweep net were reared in insect-rearing cages on a diet of milk powder and wheat bran, as described by Kumar *et al.* (2011). Hatched larvae were transferred to a 25 × 18.5 cm^2 plastic basin with a larval feed (wheat bran) that was changed daily until the larvae reached the pupal stage.

Larval bioassays. For the larval bioassay, different concentrations (5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078%) of carvacrol and CLI were prepared by dilution with acetone. The prepared concentrations were poured on a filter paper disc (in a 9.0 cm Petri plate) together with larval feed (Kumar *et al*., 2011). Acetone-treated filter paper was used as the control. Acetone was evaporated from the treated filter paper by air-drying at ambient temperature for 5 min. Twenty larvae (second instars, with larval diet) were then transferred to treated air-dried filter paper. Petri dishes containing these filter papers were incubated at 28 ± 2 °C and 75 ± 5% relative humidity (RH). The mortality of the treated larvae was recorded daily for four days. This bioassay was performed in triplicates for each concentration.

Pupal bioassays. Different concentrations of carvacrol and CLI (5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078%) were prepared for pupal assays by dilution with acetone. The produced concentrations were poured onto a filter paper disc (in a 9.0 cm Petri dish), and the acetone was removed by air drying for 5 min. Twenty pupae (2-3 days old) were placed on the filter papers and monitored for adult emergence for six days. Acetone-treated filter paper was used as a control. This bioassay was performed in triplicates for each concentration. The adult inhibition rate was calculated according to the method described by Kumar *et al.* (2011). Percentage inhibition rate (PIR) was calculated as: $PIR = \frac{Cn-Tn}{Cn} \times 100$ where Cn is the number of newly emerged houseflies in the control and Tn is the number of newly emerged houseflies in the treatment.

Statistics

The results of the different treatments were statistically analyzed using IBM SPSS for Windows version 22 (IBM, Armonk, NY, USA). Analysis of variance (ANOVA) was used to investigate the differences between treatments, and Duncan's test was used to estimate the mean differences ($\alpha = 0.05$). The lethal concentrations as well as the 50% and 90% mortality rates were calculated using SPSS v.22.

RESULTS

Antimicrobial Susceptibility Profiles

All the tested isolates were multidrug resistant, as their MDR indices were more than 20%. *S*. Enteritidis, *S*. Typhimurium exhibited resistance for 9/12 antibiotics (Table 1). However, *E. coli* was sensitive to imipenem and highly resistant to other antibiotics (Table 1). *K. oxytoka* was highly sensitive to chloramphenicol, nalidixic acid, and ampicillin. Also *P. aeruginosa* showed high resistance against all used antimicrobials except impenem (Table 1).

Table 1.
Antimicrobial susceptibility profiles of the study isolates.

	S. Entritidis	*S*. Typhimurium	*E. coli*	*K. oxytoca*	*P. aeruginosa*
	Diameter of inhibition haloes for each bacterial strain against antimicrobial agent				
ATM	R 14mm	R 0mm	R 4mm	R 12mm	R 0mm
IPM	I 20mm	R 0mm	S 28mm	I 20mm	S 22mm
CTX	R 0mm	R 0mm	R 0mm	R 0mm	R 0
AM	R 0mm	S 18mm	R 0mm	S 22mm	I 12
OT	R 0mm	R 0mm	R 0mm	R 6mm	R 6

Table 1 continuation

	S. Entritidis	*S.* Typhimurium	*E. coli*	*K. oxytoca*	*P. aeruginosa*
	Diameter of inhibition haloes for each bacterial strain against antimicrobial agent				
DO	R 2mm	R 6mm	R 2mm	R 0mm	R 4
C	S 24mm	R 0mm	R 0mm	S 42mm	R 9
S	R 2mm	R 0mm	R 4mm	R 2mm	R 6
K	R 8mm	R 10mm	R 10mm	R 8mm	I 14
NA	I 12mm	R 0mm	R 10mm	S 26mm	R 8mm
OF	R 6mm	R 0mm	R 6mm	R 2mm	R 10mm
CIP	R 10mm	R 0mm	R 10mm	R 6mm	R 8mm

ATM (Aztreonam), IPM (Impenem), CTX (Cefotaxime), AM (Ampicillin), OT (Oxytetracycline), DO (Doxycycline), C (Chloramphenicol), S (Streptomycin), K (Kanamycin), NA (Nalidixic acid), OF (Ofloxacin) CIP (Ciprofloxacin). All the bacterial isolates were evaluated for antimicrobial sensitivity to 12 different antimicrobial agents using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (Oxoid Ltd., Basingstoke, UK) according to the guidelines of CLSI (2017).

Activity of CLI against multidrug resistance bacterial certain isolates

The antimicrobial activity of carvacrol and CLI against *E. coli*, *S.* Enteritidis, *S.* Typhimurium, *P. aeruginosa*, and *K. oxytoka* was evaluated by determining the inhibitory activity using the agar dilution method. The high concentrations (10, 5, 2.5, 1.25, and 0.625 µl/ml) both of carvacrol and CLI inhibited the growth of all tested organisms. At a concentration of 0.156 µl/ml, CLI inhibited the growth of all isolates, except *S. Typhimurium* and P. aerogenosa. However, carvacrol inhibited only the growth of *K. oxytoka*, whereas the other isolates grew. Meanwhile, at a low concentration (0.078 µl/ml), all isolates grew (Table 2). *K. oxytoka* was similarly inhibited by CLI and carvacrol at various concentrations. In addition, even at low concentrations, CLI inhibited *E. coli* and S. Enteritidis when compared with free carvacrol at doubled concentrations (Table 2).

Larvicidal effects of CLI against housefly larvae

CLI exhibited significant toxicity to house fly larvae, with 100% mortality attained at a concentration of 3.82 µl/ml while pure carvacrol achieved the same result at a concentration of 4.28 µl/ml. Also, the LC_{50} for pure carvacrol was 2.54 µl/ml and 2.19 µl/ml for CLI. (Table 3).

Pupicidal inhibition activity of CLI against housefly pupae

CLI and the pure carvacrol caused 100% inhibition rate at the concentration of 50 µl/ml against pupae with LC_{50} attained at concentrations of 12.10 µl/ml and 13.20 µl/ml, respectively (Table 4).

DISCUSSION

Bacterial infections commonly cause morbidity and mortality in humans and animals globally (WHO, 2010), and can even cause food deterioration (Srinivasa & Tharanathan, 2007). The main strategy for managing these bacterial infections is the use of antibiotics (Zaffiri *et al.*, 2012). However, improper use of these chemicals has contributed to the development and spread of multidrug resistant pathogens (English & Gaur, 2010). As a result, antibiotic-resistant organisms pose a significant threat to animal and public health. Therefore, it is critical to conduct research and develop novel chemicals with antibacterial properties that do not harm animal or human cells (Ling *et al.*, 2015). Essential oils and their constituent small molecules offer a good alternative therapeutic option for microbial diseases (Basri *et al.*, 2014; Raut & Karuppayil, 2014).

Musca domestica is a mechanical vector of more than 100 pathogens (bacteria, fungi, viruses, and parasites), some of which cause severe diseases in humans and domestic animals. Chemical pesticides, particularly pyrethroids, are frequently used to control houseflies (Shah *et al.*, 2015). Unfortunately, resistant pyrethroid houseflies have emerged. Furthermore, chemical insecticides are hazardous to the environment, domestic animals, and humans (Scott *et al.*, 2013). Therefore, it is necessary to develop new materials that use biodegradable and target-specific insecticides to control flies in a manner that is safe for animals, humans, and the environment.

Several studies have shown that essential oils are effective in controlling houseflies (Chauhan *et al.*, 2016; Benelli *et al.*, 2018; Pavela *et al.*, 2018). The antibacterial and an-

Table 2.
Antibacterial activity of carvacrol and carvacrol-loaded invasomes (CLI).

Isolates Concentrations	*S.* Entritidis	*S.* Typhimurium	*E. coli*	*K. oxytoca*	*P. aeruginosa*
			Inhibition of bacterial growth		
Carvacrol 10 µl/ml	+	+	+	+	+
CLI 10 µl/ml	+	+	+	+	+
Carvacrol 5 µl/ml	+	+	+	+	+
CLI 5 µl/ml	+	+	+	+	+
Carvacrol 2.5 µl/ml	+	+	+	+	+
CLI 2.5 µl/ml	+	+	+	+	+
Carvacrol 1.25 µl/ml	+	+	+	+	+
CLI 1.25 µl/ml	+	+	+	+	+
Carvacrol 0.625 µl/ml	+	+	+	+	+
CLI 0.625 µl/ml	+	+	+	+	+
Carvacrol 0.313 µl/ml	-	+	-	+	-
CLI 0.313 µl/ml	+	+	+	+	+
Carvacrol 0.156 µl/ml	-	-	_	+	-
CLI 0.156 µl/ml	+	-	+	+	-
Carvacrol 0.078 µl/ml	-	-	-	-	-
CLI 0.078 µl/ml	-	-	-	-	-

+ means inhibition of bacterial growth
- means growth of bacteria

Table 3.
Larvicidal activity of pure carvacrol and Carvacrol-loaded invasomes against house fly larvae.

Concentrations µl/ml	Carvacrol Larval Mortality percentage	Carvacrol-loaded invasomes Larval Mortality percentage
50 µl/ml	100 ± 0.00*	100 ± 0.00*
25 µl/ml	100 ± 0.00*	100 ± 0.00*
12.50 µl/ml	100 ± 0.00*	100 ± 0.00*
6.25 µl/ml	100 ± 0.00*	100 ± 0.00*
3.125 µl/ml	63.0 ± 5.70*	74.0 ± 4.18*
1.56 µl/ml	26.0 ± 4.18*	33.0 ± 2.74*
0.78 µl/ml	14.0 ± 4.18	19.0 ± 4.18*
LC_{50}	2.54 µl/ml	2.19 µl/ml
LC_{90}	4.28 µl/ml	3.82 µl/ml
Acetone (Negative control)	2.00 ± 2.74	2.00 ± 2.74
Deltamethrin 2 ml/l	12.0 ± 2.74	12.0 ± 2.74

(*) Significant for negative control

Table 4.
Pupicidal activity of pure carvacrol and Carvacrol-loaded invasomes against house fly pupae.

Concentrations µl/ml	Percentage inhibition rate (PIR) of carvacrol	Percentage inhibition rate (PIR) of carvacrol-loaded invasomes
50 µl/ml	100 ± 0.00*	100 ± 0.00*
25 µl/ml	82.5 ± 4.92*	90.3 ± 4.30*
12.50 µl/ml	54.3 ± 6.34*	53.2 ± 5.25*
6.25 µl/ml	40.2 ± 5.69*	40.2 ± 4.12*
3.125 µl/ml	22.8 ± 2.80*	23.9 ± 2.94*
1.56 µl/ml	2.16 ± 2.96	3.33 ± 3.03
0.78 µl/ml	0.00 ± 0.00	0.00 ± 0.00
LC_{50}	12.2	12.1
LC_{90}	24.9	22.3
Acetone (Negative control)	0.00 ± 0.00	0.00 ± 0.00
Deltamethrin 2 ml/l	0.00 ± 0.00	0.00 ± 0.00

(*) Significant for negative control

tioxidant properties of monoterpenoids, such as carvacrol, make them an excellent alternative to commercially available chemical compounds for pest control and regular bactericides (Didry *et al.*, 1994; Undeger *et al.*, 2009). Carvacrol has been shown to be acutely toxic to a variety of invertebrate pests, including insects, flies, and mosquitoes (Cetin *et al.*, 2009; Dolan *et al.*, 2009; Lei *et al.*, 2010), anti-parasitic (Force *et al.*, 2000), and to cause little harm to mammals, fish, and other non-target organisms, while also biodegrading or disintegrating efficiently in the environment (Aboelhadid *et al.*, 2013; Sinthusiri & Soonwera, 2014).

In the current study, carvacrol-loaded invasomes (CLI) were prepared and compared to pure carvacrol against isolates of *E. coli*, *S. enterica* Enteritidis, *S. enterica* Typhimurium, and *P. aeruginosa* that were multidrug resistant, as well as against housefly resistant to insecticides.

The results showed that at a concentration of 0.625 µl/ml and more carvacrol and CLI, stopped the growth of all organisms examined. CLI inhibited the growth of only *E. coli* and *S. enterica* Enteritidis at a dose of 0.156 µl/ml or less, but pure carvacrol had no impact even at a two-fold concentration. Similarly, Bnyan *et al.* (2014) determined the antibacterial activity of carvacrol against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Serratia* spp., and the results revealed that all examined bacterial isolates showed significant growth inhibition at different concentrations, except *Pseudomonas aeruginosa*. Also, Burt *et al.* (2007) found that carvacrol is efficient at inhibiting the growth of *S. enterica* serotype Enteritidis on agar as well as eliminating these bacteria from raw chicken surface.

Kamimura *et al.* (2014) found that carvacrol inclusion complexes with hydroxypropyl-beta-cyclodextrin (HPB-CD) had stronger antibacterial activity against *Escherichia coli* K12 and *Salmonella enterica* serovar Typhimurium LT2 than pure carvacrol. In addition, Persico *et al.* (2009) discovered significant antibacterial activity of nanocomposite films based on low-density polyethylene containing carvacrol compared to pure carvacrol. Similarly, Liu *et al.* (2022) prepared a carvacrol nanoemulsion to overcome the instability and water insolubility of commercial carvacrol and then tested its antibacterial activity.

These findings suggest that CLI disrupted the structure and permeability of bacterial cells, allowing them to permeate the contents of the bacterial cells and, as a result, greatly outperform commercial carvacrol in terms of antibacterial activity. The antibacterial properties of carvacrol have been attributed to its ability to alter bacterial membrane permeability and trigger the leakage of potassium, phosphate, and protons (Lambert *et al.*, 2001).

Carvacrol and CLI showed significant larval *Musca* mortality even at low concentrations with LC_{50} reached at concentrations of 2.54 and 2.19 µl/ml, respectively, both also showed a significant PIR at low concentration (3.125 µl/ml). Similarly, Xie *et al.* (2019) assessed the insecticidal ac-

tivity of carvacrol against the housefly (*M. domestica*) and found considerable toxicity with an LC_{50} attained at concentration of 0.03 µl/l for contact toxicity and 2.78 µl/l for fumigation toxicity. Also, carvacrol showed a percentage inhibition rate (PIR) of 29.5% at concentration of 0.025 µl/l and 81.8% at concentration of 1.25 µl/l for the contact toxicity and fumigation assay, respectively (Xie *et al*., 2019).

The insecticidal activity of carvacrol can be explained by a number of mechanisms, including binding to the nicotinic acetylcholine receptor found in the housefly central nervous system (Itier & Bertrand, 2001; Jeschke & Nauen, 2008), targeting the gamma-aminobutyric acid receptor (Garcia *et al*., 2006), an octopamine receptor (Gross, 2010), and a tyramine receptor (Enan, 2005), and inhibition of acetylcholinesterase (Anderson & Coats, 2012). Tong *et al*. (2013) used [14C]-Nicotine binding assays with *M. domestica* nicotinic acetylcholine receptors (nAChRs) to explain the mode of action of carvacrol against *M. domestica* and they discovered that carvacrol binds to housefly nAChRs at a different binding site than nicotine and acetylcholine, which may support the idea that carvacrol's insecticidal effect involves nAChRs as a potential target.

CLI achieved better antibacterial and insecticidal activities than pure carvacrol, which can be attributed to the invasome formulation, which increased the penetration capacity of the loaded carvacrol. This was supported by the HPLC data, which showed that ticks treated with the CLI formulation had a significantly ($p < 0.001$) higher penetration than carvacrol by 3.86 folds (Gamal *et al*., 2023). The combination of ethanol and terpene in the invasomal bilayer breaks down hydrogen bonds between ceramides in the insect cuticle, increasing the space available for medicinal assimilation (Ahmed *et al*., 2019; Ahad *et al*., 2011).

We were limited by the use of invasomes without carvacrol as a control. The invasomes were prepared in a 10 ml organic solution of chloroform and methanol (3:1), in which carvacrol (10 mg), cineole (1% v/v), cholesterol (0.15% w/w), and phospholipid (3% w/w) were dissolved. Except for carvacrol, all ingredients were added at a range of 1%, which had little effect as an insecticide or antibacterial agent. Moo *et al.* (2021) found that 1,8-cineol possessed bactericidal effect against carbapenemase-producing *Klebsiella pneumoniae* (KPC-KP) at 28.83 mg/ml. Furthermore, cineole has been shown to be toxic to *Musca domestica* at concentrations of 4 µl/l (Rossi & Palacios, 2015). The effective concentration of CLI against larvae was 6.25 µl/ml and that against pupae was 50 µl/ml. This implies that the concentration of cineol used to prepare the CLI was higher than that reported by Rossi & Palacios (2015). This indicates that cineol had no discernible impact on the study. As a result, we did not use individual invasome components as controls.

In conclusion, the carvacrol-loaded invesomes demonstrated significant antibacterial activity, particularly against MDR isolates, as well as insecticide activity against their mechanically transmitted vector, the housefly.

DECLARATIONS

Competing interests statement

The authors declare that they have no competing interests.

Ethics statement

Not applied

Author contributions

Conceptualization: SMA, FIAE, AG; Data curation: SAQ, ASA, AOH; Formal analysis: SMI, NH A; Funding acquisition: SAQ, ASA; Investigation: MAY, SMI, AG; Methodology: MAY, FIAE; Supervision: SMA, ASA, KAMS; Validation: SAQ, SMA, AOH; Visualization: MAY, SMI, KAMS; Roles/Writing - original draft: MAY, SMI, FIAE; Writing - review and editing: ASA, SMA.

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SHORT COMMUNICATION

The 2022 Crimean-Congo Hemorrhagic Fever outbreak in Iraq

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ABSTRACT. Crimean-Congo Hemorrhagic fever (CCHF) is an endemic, zoonotic, viral, and tick-borne disease that causes hemorrhagic symptoms. The disease has been reported in Iraq since 1979 comprising six cases between 1989 and 2009. Subsequently, 11, 3, and 33 cases were reported in 2010, 2018, and 2021, respectively. This study describes the 2022 Crimean-Congo Hemorrhagic fever virus (CCHFV) outbreak in different Iraqi governorates. In the 2022 outbreak, 212 cases of human CCHFV were reported between January 1 and May 22. These included 97 (46%) cases confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and 115 (54%) suspected cases. Most human cases have been reported for livestock breeders and butchers. Approximately half of the confirmed cases (48%) were reported in the Dhi Qar governorate, whereas the others were reported in Missan, Muthanna, Wasit, Diwaniya, Karkh/Baghdad, Rusafa/Baghdad, Kirkuk, Basra, Najaf, Nineveh, Babylon, and Karbala. Patients with CCHFV showed symptoms of the pre-hemorrhagic phase, including sudden fever, anorexia, vomiting, diarrhea, headache, nose bleeding, and abdominal and joint pain. Later, hemorrhagic symptoms started with unexplained bleeding lesions, ranging from small petechiae to large hematomas. The factors responsible for the re-emergence of CCHFV included a shortage of veterinary services during the COVID-19 pandemic and illegal slaughtering of animals outside the abattoirs. This led to massive tick infestations that acted as viral vectors.

In conclusion, this is the first report documenting the 2022 CCHFV outbreak in Iraq, with a total of 212 patients and 27 overall deaths, including 13 laboratory-confirmed cases. The authors recommend improving veterinary services provided to farmers. Additionally, future studies need to be done including the sero-epidemiology and molecular studies on local livestock and ticks to understand their roles in circulating the virus to humans.

Keywords: CCHFV; Iraq; Dhi Qar; Tick; Zoonotic

INTRODUCTION

Crimean-Congo Hemorrhagic fever (CCHF) is a severe zoonosis with a mortality rate of up to 40%. This is a tick-borne viral disease. This pathogen belongs to the *Nairoviridae* family within the Bunyavirales order, formerly Bunyaviridae. It is a single-stranded, spherical enveloped RNA virus (Kuehnert *et al.*, 2021; Hawman & Feldmann, 2018; Emmerich *et al.*, 2018). CCHF virus is commonly transmitted to humans through tick bites, tick crushing, or contact with infected human secretions, such as blood and tissues or blood from symptomatic infected animals (Moraga-Fernández *et al.*, 2021; Monsalve *et al.*, 2021; Gruber *et al.*, 2019).

The disease was first reported in Crimea in the 1940s by Soviet military workers after occupying Crimea (Casals, 1969). Then, an antigenically identical virus was diagnosed in the Belgian Congo (currently named the Democratic Republic of Congo) in the 1960s. Hence, the disease was named Crimean–Congo Hemorrhagic fever. Until the Autumn of 1979, Iraq was free from CCHF, but it has been endemic for approximately 50 years. The first CCHF case was recorded on September 3, 1979, and subsequently nine patients were reported (Al-Tikriti *et al.*, 1981; Al Salihi *et al.*, 2023).

The causative agent was isolated from the patient's blood and *post mortem* liver samples and was antigenically closely related to other members of the CCHF virus group (Tantawi *et al.*, 1980). Since the fourth quarter of 1979, Iraq has been considered a CCHF endemic country, as it is located at the heart of eastern Mediterranean countries. Since then, multiple outbreaks of CCHF have been reported between 1989 and 2009. Additionally, 11 cases, 3 fatal cases, and 33 confirmed cases (including 13 deaths and 39% fatalities) were reported in 2010, 2018, and 2021, respectively. Sixteen cases have been reported in the Dhi Qar governorate, resulting in seven deaths in 2021 (Aamir, 2022).

In 2015, Iraq had an enormous population of large and small ruminants, estimated at 16.432, 1.885, 3.172, 0.395, and 0.11 million heads of sheep, goats, cattle, buffalo, and camels, respectively, according to the Food and Agriculture Organization (FAO) Statistics Division (FAOSTAT, 2015). Annually, the products of these animals, such as milk, meat, wool, and skin, contribute to approximately 50% of Iraq's agricultural gross domestic product (Al-Salihi, 2012). Ticks are important vectors or pathogenic agents

and have also led to health problems in Iraqi livestock, such as stress, tick-borne diseases, tick paralysis, dermatitis, anemia, and secondary infections (Al-Salihi *et al.*, 2018). According to Hoogstraal & Kaiser (1958), there are 21 ticks from several genera in Iraq, including *Argas*, *Ornithodoros*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus*. Many researchers have reported heavy tick infestations in livestock during various seasons; however, higher infestations were reported in July and lower in May, according to previous studies (Hassan & Simpson, 1981; Shamsuddin & Mohammad, 1988).

Previous studies have acknowledged that ticks, mainly Hyalomma species, which is the principal vector of CCHF, and *Rhipicephalus* and *Haemaphysalis* (Hasson & Al-Zubaidi, 2014; Mohammad, 2016; Mallah & Rahif, 2016), commonly infest animals in Iraq. A literature review of various Medline platforms including PubMed, ProQuest, EBSCOhost, Web of Science, and Ovid regarding the distribution of tick species showed a scarcity of publications on CCHF outbreaks, especially in Iraq. This suggests that limited research has been conducted on CCHF outbreaks in Iraq, highlighting the need for further investigation and surveillance.

Therefore, this study was designed to study the occurrence of the 2022 CCHF Crimean-Congo Hemorrhagic fever outbreak in Iraq, based on its relation to heavy tick infestation in livestock and hospital records.

MATERIALS AND METHODS

In early January 2022, suspected human CCHF cases were reported to the World Health Organization (WHO) by the authorities of the Iraqi Ministry of Health. A cross-sectional study was conducted on 212 human CCHFV cases reported in various Iraqi Governorates. Data on this outbreak, including information on all patients, were extracted from their medical records and analyzed. The number and percentage of infected humans are reported for each governorate. Based on the World Health Organization's resources, the standard definition of a CCHF-suspected case is, "An individual with a quick onset of illness, with high grade fever of > 38.5 ºC, for more than 72 hours, for < 10 days, in a CCHF endemic region, who was in contact with livestock." During this outbreak, numerous criteria were considered to diagnose the suspected cases of CCHF. These included sudden fever accompanied by thrombocytopenia (< 50.000 / mm3), hemorrhagic symptoms, including nosebleeds (epistaxis), gum bleeding, petechial or purpuric rashes, hematemesis, hemoptysis, melena, ecchymosis, hematuria, and other hemorrhagic manifestations.

All CCHFV-suspected patients who showed these symptoms were hospitalized in separate CCHFV wards and Intensive Care Units. Blood samples were collected from all (212) cases reported during the outbreak. The samples were sent immediately to the Central Public Health Laboratory (Baghdad) and handled under maximum biological containment conditions because the CCHF patient samples present an extreme biohazard risk. Active CCHFV infection was detected by amplification of CCHFV 5RNA or by capturing CCHFV-specific IgM or IgG titrations following the acute phase of infection (WHO, 2019; WHO, 2018; Gupta *et al.*, 2017; Drosten *et al.*, 2003).

In Iraq, Scenario 1 was used to detect CCHF cases. This protocol was established by the WHO R&D Blueprint, a roadmap priority to serve as a bridge to target the product profile (TPP) for diagnosing CCHF. These scenarios were established for acute and early detection of the disease during outbreaks. The WHO and CCHF Roadmap strategic priorities were updated and validated in 2018 and 2019 for rapid, simple-to-use, and easily accessible diagnostics to enable effective medical intervention and reduce deaths and morbidity from CCHF.

Serum samples were inactivated prior to nucleic acid extraction and amplification to ensure specimen handling safety. Viral RNA was extracted from the serum samples according to the manufacturer's instructions (Geneaid, South Korea). The RT-PCR oligonucleotide sequences and primers for the detection of CCHFV genes were performed according to the WHO-validated kit (CCHFV RNA, validated for Eurasian clades IV-VII). The RT-PCR test provides the maximum detection sensitivity for active infection at the earliest possible time (WHO, 2019; WHO, 2018; Gupta *et al.*, 2017; Yilmaz *et al.*, 2008; Drosten *et al.*, 2003).

Multiple field visits were conducted during the CCHFV outbreak to examine tick distribution, especially in areas where disease cases were reported.

RESULTS AND DISCUSSION

The total number of cases was 212, of which 165 (80%) were reported during April and May 2022. Among the 212 cases, 115 were suspected and 97 were laboratory-confirmed. Additionally, there were 27 deaths, of which 13 were laboratory-confirmed cases, with a case fatality ratio (CFR) of 13% (13/97). The history of these cases showed that most were people who had direct contact with animals, such as livestock, breeders, and butchers. Additionally, more than half of the confirmed patients were 15–44 years old (n = 52; 54%) and male (n = 60; 62%).

The results of RT-PCR showed that approximately 50% of the confirmed cases (n = 47; 48%) were reported in Dhi Qar, including eight deaths. The rest of the cases were reported from Missan (13), Muthanna (7), Wassit (6), Diwaniya (4), Karkh/Baghdad (4), Kirkuk (3), Basra (3), Najaf (3), Nineveh (3), Rusafa/Baghdad (2), Babylon (1), and Karbala (1) (Figure 1).

Most patients experience a sudden onset of fever and bleeding from the nose accompanied by anorexia, vomiting, diarrhea, headache, abdominal pain, and joint pain during the first stage. Later, the patient had a sudden onset

of hemorrhagic manifestation, starting with unexplained bleeding and lesions ranging from small petechiae to large hematomas. Bleeding in the uterus and gastrointestinal, respiratory, and urinary tracts has also been observed in some patients. Additionally, the deadliest CCHFV cases suffered from multifunctional failure, including hepatitis, rapid kidney deterioration, and sudden liver or respiratory failure that led to death.

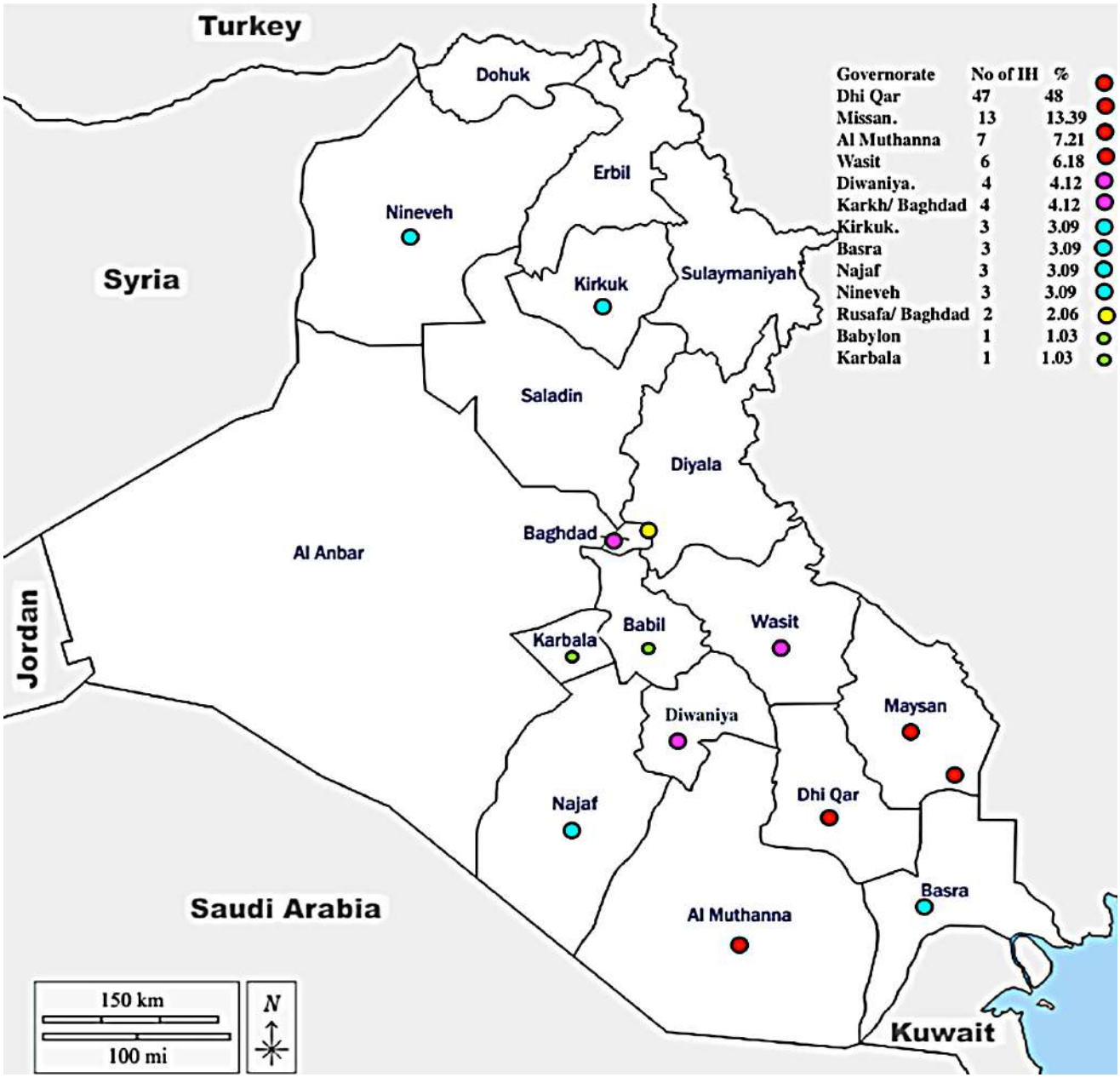

Figure 1.
Map of Iraq with the number of Infected Human (N° of IH) of CCHF and the percentage of infection in different Iraqi governorates during the 2022 outbreak.

Field visits to farmers during the CCHFV outbreak have revealed an enormous number of ticks. Most animals suffered from heavy tick infestations, which promoted the distribution of tick-borne diseases (Figure 2). Acaricide campaigns were initiated in villages, rural areas, and areas where farm animals were sold from May 1, 2022, to May 31, 2022, to control tick multiplication by all veterinary hospitals in the Iraqi governorates. Despite these procedures, CCHFV cases continued in different regions of Iraq, especially the DhiQar Governorate.

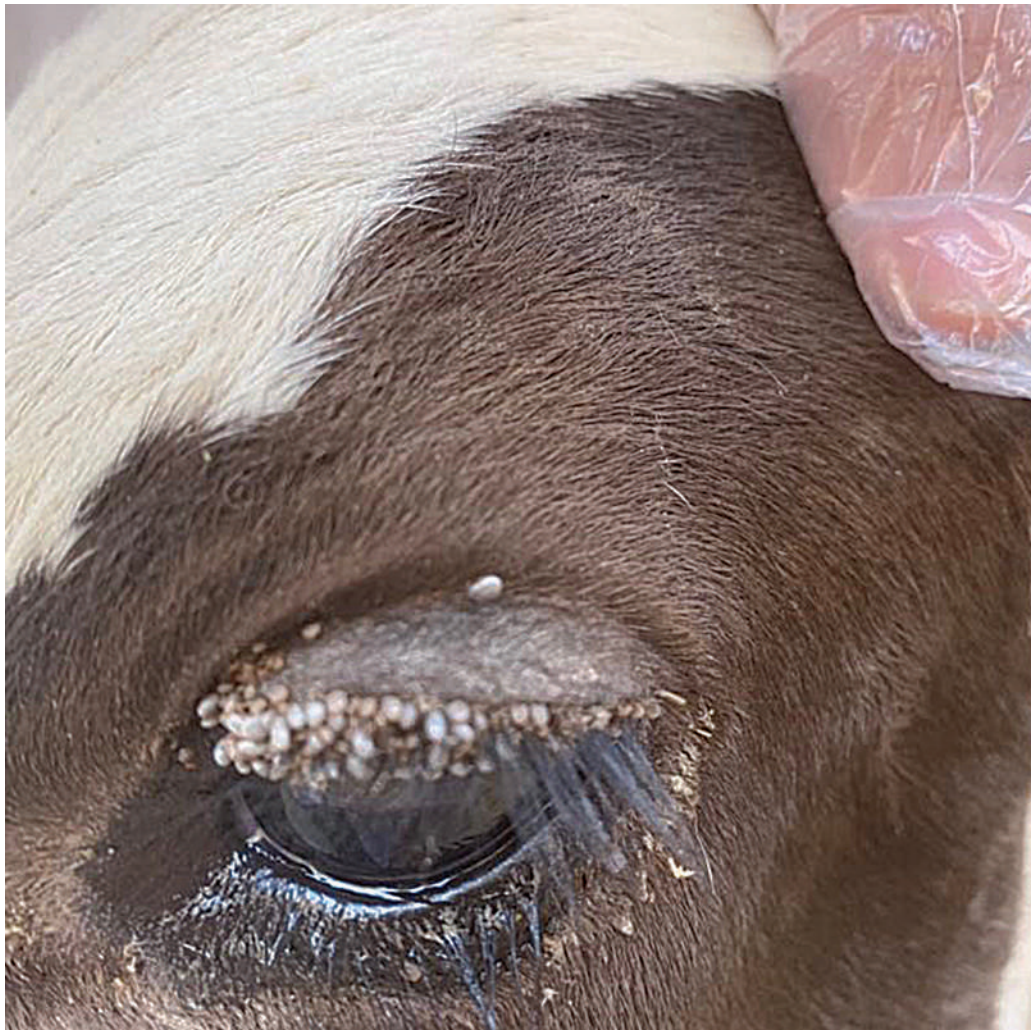

Figure 2.
A heavily infested calf with ticks from an area where a patient with CCHF was reported.

The current outbreak created fear in the people, especially those who worked with the livestock, and they called it "the deadly nose-bleed fever disease," because most patients showed bleeding from the nose.

The current CCHFV outbreak in Iraq between January 2022 and June 2022 was primarily associated with tick bites. According to WHO's representative in Iraq (https://www.al-monitor.com/originals, 2022), huge tick multiplication was one of the postulations for the current attack. The heavy spread of ticks occurred because of the absence of livestock spraying campaigns during the coronavirus pandemic in 2020 and 2021.

The heavy tick population is a well-recognized risk factor, and its features are similar to those of previous CCHFV outbreaks scrutinized in Iraq (Ali, 2020) and worldwide (Sánchez-Seco *et al.*, 2021; Yagci-Caglayik *et al.*, 2014; Mertens *et al.*, 2013; Ozkurt *et al.*, 2006; Karti *et al.*, 2004; Chapman *et al.*, 1991). However, global warming has also extended the period of tick multiplication. Geographically, Iraq is an eastern Mediterranean country where CCHFV

is endemic, and outbreaks are becoming more frequent. Several CCHFV outbreaks were reported between 1989 and 2009. Furthermore, 11 and 33 confirmed cases, including 13 deaths (39% fatalities), were reported in 2010, 2018, and 2021. Additionally, 16 cases were reported in the DhiQar governorate, resulting in seven deaths in 2021. There are similarities between the 2022 and 2021 CCHF outbreaks. In 2021, the disease was reported in week 19, with 45 cases resulted in nine deaths, including five laboratory-confirmed deaths. The 2021 outbreak revealed that the number of cases peaked at weeks 27 (four cases) and 38 (four cases). Nonetheless, most of the 2021 cases were from DhiQar (10), Ninewa (2), Erbil (3), Baghdad (3), Babel (2), Diyala (1), and Anbar (1). Regarding sex, the ratio of males to females was 1.2:1, indicating that more males than females were affected by the disease. However, no cases have been reported among healthcare workers. The 2021 outbreak was recorded in the southern governorates during the summer months, from July to November. However, the authorities mentioned that the illegal slaughtering of animals outside the abattoirs contributed to the spread of the disease.

The clinical signs reported in the current outbreak were similar to those reported by other researchers (Appannanavar & Mishra, 2011; Yilmaz *et al*., 2008; Ergonul, 2006). Most cases have an incubation period ranging from three to seven days, and they develop severe early clinical signs, followed by various hemorrhagic lesions (Avšič-Županc, 2007; Golden *et al*., 2022). The current outbreak is a recurrent remerging of CCHFV infection in Iraq. However, the number of recorded cases was unprecedented. The high number of infected individuals is attributed to several factors including the lack of veterinary services during the COVID-19 pandemic. Additionally, a new retirement law at the age of 60 ended the jobs of many veterinarians, which led to reduced veterinary services provided to farmers by government sectors. This adversely affects livestock health. Furthermore, since 1989, the Iraqi government has stopped employing graduate veterinarians in different sectors, especially in the Ministry of Agriculture. These actions have created vacant places and an apparent lack of veterinary services for farmers in most Iraqi governorates.

CONCLUSION

A new outbreak of CCHF was reported in Iraq in 2022 with 212 patients, including 115 suspected and 97 laboratory-confirmed cases, with 27 overall deaths, including 13 laboratory-confirmed cases (case fatality ratio, 13%; 13/97). RT-PCR was performed to confirm the diagnosis. Authorities must provide veterinary services to control ticks, the CCHFV vector, and regulate the illegal slaughtering of animals outside abattoirs. Seroepidemiological and molecular studies must be conducted in livestock to understand their role in circulating the virus in humans. More studies are needed on ticks, which are viral vectors.

DECLARATIONS

Competing interest statement

The authors declare that they have no conflicts of interest.

Ethics statement

This study was approved by the Research and Animal Ethics Committee of the College of Veterinary Medicine, Al Muthanna University, 2022.

Author contributions

Karima provided the concepts, data analysis, and writing of the manuscript; Mohammed worked with data collection and analysis; Zakaria worked with data collection and analysis; and Hussain revised the manuscript and analyzed the data.

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SHORT COMMUNICATION

Effects of selling through an auction market on physiological variables in beef calves

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ABSTRACT. In Chile, selling cattle through auction markets is common, and involves various stressful events. The objective of this study was to determine the effects of selling weaned beef calves through an auction market on indicators of physiological stress. Considering that transportation is invariably linked to any commercialization procedure, we compared the effects of exposing calves to transport and sale through an auction market versus transporting them directly between farms. Twenty calves of 175 (+/-37.5) kg live weight were transported from their origin farm to an auction market (2.5 h journey), where 10 calves were unloaded and exposed to regular handling within the market (unloading, sorting, regrouping, penning, sale yard showing) and thereafter loaded and transported back to the farm (TM). The remaining 10 calves were not unloaded at the market and were immediately transported back to the farm, simulating a direct sale (TD). Data collection (blood samples, body temperature, and live weight) was performed at the farm in both groups before loading (initial, 0 h), at the end of the process, after unloading TM calves back on the farm (after unloading, 12 h), and during recovery at 24, 48, and 72 h after the initial sampling. We used mixed-effects models to compare treatments and samplings. No differences were found between treatments in terms of live weight loss, maximum eye temperature, plasma β-hydroxybutyrate, and serum cortisol concentrations. The rectal temperature was higher ($p < 0.05$) in TM after treatment and remained higher ($p > 0.05$) in the samples than in TD. Calves that passed through the market showed higher ($p < 0.05$) plasma CK activity after unloading (12 h) than did those that did not. The mean glucose concentration increased ($p < 0.05$) at 12h and remained higher than the initial value (0 h) in both the calf groups. This was a preliminary study, and according to the results, experiments should be repeated to test more animals under different situations and premises.

Keywords: cattle, transport, fasting, marketing, stress indicators, welfare.

INTRODUCTION

The marketing process is stressful for calves because they are taken away from their environment and often weaned immediately before loading, transported, and unloaded in a new environment. In addition, during marketing, cattle are deprived of food and water, usually from the moment they are herded before being transported, until they arrive at their destination. Selling animals through auctions also increases their handling and exposes them to a greater number of interactions with humans (Weeks *et al.*, 2002; Gregory *et al.*, 2009).

As in many other Latin American countries, selling animals through livestock auction markets is still common in Chile, particularly for calves sold for backgrounding purposes (Gallo & Tadich, 2008). In 2020, 900,185 cattle were auctioned in Chilean markets, 33% of which were calves (INE, 2021). Studies conducted in Chilean auction markets using behavioral indicators have shown that calf welfare is negatively affected, mostly associated with poor handling by market staff (De Vries, 2011; Bravo *et al.*, 2019, 2020).

The objective of this study was to determine the effects of selling weaned beef calves through an auction market on physiological variables indicators of stress. The hypothesis states that the mean values of physiological indicators of stress would be higher in calves passing through the auction market than in those sold directly.

MATERIALS AND METHODS

Animals and study design

Twenty Angus calves (black and red) of both sexes (15 females and 5 males), weaned a month before the experiment, with an approximate age of 7 (± 1) months, weighing 175 kg (± 37.5), and clinically healthy were chosen for the study. All calves were born and raised on the same farm where the experiments were conducted. They were kept on pasture during the day, with *ad libitum* access to water. During the night, calves were placed in a barn and received 1 kg/head of sugar beet pulp pellets (87% dry matter and 15% protein) and hay (*Lolium perenne*) *ad libitum*. The experiment was conducted in southern Chile during winter (2-10°C and 85-95% humidity during the experimental period).

The calves were divided according to sex and weight into two homogeneous groups. They were loaded and transported in the same truck to an auction market for 2.5 hours.

Each group was placed in a separate pen with a similar space allowance within the truck (approximately 1 m^2 per 270 kg). One group was unloaded at the market and exposed to all regular handlings within the market (TM), including classifying, penning, auctioning, re-penning, and loading, to be transported back to the farm of origin. The other group was not unloaded at the market and was immediately transported back to the farm of origin, simulating a direct sale between farms (TD). At the farm, the TD group was unloaded and maintained in a pen without food or water until the TM group arrived to preserve the same fasting time in both groups. The total fasting time was 12 h, including 5 h of transportation to and from the auction market.

Data collection

Blood samples were taken from all calves five times during the experiment, while restrained in a chute at the farm, by coccygeal venipuncture using a Vacutainer® (needle 20G x 1"). Initial sampling (0h) was performed before loading the calves for transport in both groups. The next sampling also took place for both groups simultaneously, coinciding with the arrival and unloading of the calves that passed through the auction market 12 h after the initial sampling. This sampling (12 h) included the possible effects of 5 h of total transportation time (to the auction market and back to the farm in both groups), plus fasting for 7 h and exposure to several handling procedures during marketing in TM, including one extra loading and unloading. Further sampling was performed during the recovery period of both groups of calves at 24, 48, and 72 h after the initial sampling. Body temperature was measured, and the live weight of the calves was recorded at each sampling time.

Blood variables

Three collection tubes were used during blood sampling: a tube without additives was used for measurements of cortisol serum concentrations, and a tube with EDTA was used to measure creatine kinase activity (CK) and the concentration of β-hydroxybutyrate (β-HB). A third collection tube containing sodium fluoride (NaF) was used for glucose measurements. All blood samples were immediately placed on ice and transported to the Veterinary Clinical Pathology Laboratory of Universidad Austral de Chile. Frozen serum samples were sent to PetLab Laboratory, located in Santiago, Chile, and cortisol concentrations were determined using chemiluminescent immunoassay (CLIA). Plasma glucose concentration was determined using the GOD-PAP test. The plasma concentrations of β-HB were determined by an enzymatic technique using 3-hydroxybutyrate dehydrogenase, and plasma CK activity was measured using the IFCC and ECCLS kinetic methods.

Body temperature

The Maximum Eye Temperature was obtained by capturing infrared images of the left eye at approximately 0.5 m distance (90 ° angle from the individual) using a thermal camera (FLIR i5, FLIR Systems, Wilsonville, OR, USA). The camera was calibrated with an emissivity of 0.95, according to the information provided by the manufacturer. Image analysis was performed using FLIR Tools 3.1 (FLIR Systems, Wilsonville, OR, USA). The atmospheric temperature and relative humidity were included in the calculations. The rectal temperature was measured after blood samples were collected using a digital thermometer.

Live weight

All calves were individually weighed at all samplings, after all other measurements were obtained, using a mechanical cattle scale (Romana, 0.5–1000 kg). To control for weight differences between animals, the percentage of weight variation in relation to the initial weight before loading was estimated for each animal weighed during each sampling period.

Data analysis

For descriptive analyses, variables were shown as mean and standard error (SE) using Microsoft Excel 2016. To assess the effect of direct sale (TD) or sale through an auction market (TM) on the dependent variables, mixed-effect models were built, including treatment and sampling time as fixed effects and calf as a random effect. Data were analysed using the "lme4" statistical package and multiple comparisons were explored using a Tukey's adjustment included in the "lsmeans" function in R Studio software version 3.2.2; $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

To the best of our knowledge, this is the first study to measure physiological variables related to stress in calves in a real commercial situation after transporting and passing through an auction market. To date, studies have mainly reported descriptive conditions in the markets related to behavioral indicators of animals, handling by stockpersons, and infrastructure (Weeks *et al*., 2002; Gregory *et al*., 2009; Romero *et al*., 2017; Bravo *et al*., 2019). In this study, both groups had equal transport and fasting times. However, the potential stress associated with regular handling during the marketing process is present only in calves that pass through the market.

Calves that passed through the market showed higher plasma CK activity after unloading (12 h) than those that did not ($p < 0.05$; Table 1). CK activity has been shown to increase in steers transported (3 or 16 h) as well as in steers confined to a pen for the same time (Tadich *et al*., 2003). Bravo *et al.* (2018) also reported increases in CK concentrations in calves transported for 3 h. Increases are related to exercise and/or muscle damage caused by fighting, mounting, and bruising (Knowles *et al*., 2014). Therefore, the higher CK activity in TM calves could be due to handling and movement within the market premises, likely mixing and interacting with other cattle, and hitting themselves with the infrastructure (Weeks *et al*., 2002; Gregory *et al*., 2009;

Romero *et al*., 2017; Bravo *et al*., 2019). All mean CK activity values were higher than reference values (Wittwer, 2012). Interestingly, the mean CK activities in calves transported directly to the farm were significantly lower ($p < 0.05$) at 48 and 72 h than those initially (0 h). This could indicate that CK values were already high at the start of the experiment, probably because of herding and sampling, which has also been found in other experiments with calves of similar age kept under extensive conditions and sampled before loading for transport (Werner *et al*., 2013).

When comparing plasma glucose concentrations at different sampling times, an increase was found between 0 and 12 h in both groups of calves ($p < 0.05$), without differences between treatments (Table 1). In some cases, the concentrations were even higher than the normal reference values for the specie (2.5-4.1 mmol/L; Wittwer, 2021). The before-loading concentrations (0 h) recovered 72 h after loading in both groups, with non-significant interactions between groups and sampling times. Increases in blood glucose concentrations have been previously reported in calves and steers after transport and fasting owing to confinement (Tadich *et al*., 2003; Earley *et al*., 2006; Werner *et al*., 2013; Bravo *et al*., 2018). The increase in the glucose concentration observed in the present study may be related to the primary response to stress (Knowles *et al*., 2014).

Mean cortisol concentrations did not differ between

Table 1.
Means and standard errors (±SE) of the blood variables related to stress in only transported (TD) and transported and marketed (TM) calves at different sampling times (Initial, After Unloading (AU), and during recovery at 24, 48, and 72 h after the initial sampling).

Sampling times	CK (U/L)			Glucose (mmol/L)			β-HB (mmol/L)			Cortisol µg/dL		
	TD	TM	p	TD	TM	p	TD	TM	p	TD	TM	p
Initial 0h	302±31^{a}	245±12^{a}	ns	3.6±0.1^{a}	3.4±0.1^{a}	ns	0.28 ±0.04a,b	0.19±0.02a	ns	1.5±0.2^{a,b}	1.4±0.2^{a,b}	ns
AU 12h	298±37^{a}	442±54^{b}	*	4.3±0.2^{b}	4.1±0.1^{b,e}	ns	0.19±0.02a	0.27±0.04a	ns	2.0±0.4^{a,b}	2.4±0.3^{b,c}	ns
Recov 24h	220±17^{a,b}	288±25^{a}	ns	4.4±0.2^{b}	4.4±0.1^{b,c}	ns	0.36±0.05b	0.22±0.03a	ns	2.4±0.5^{b,c}	1.6±0.3^{a,b}	ns
Recov 48h	169±29^{b}	164±08^{c}	ns	4.1±0.1^{b,c}	3.9±0.1^{d,e}	ns	0.18±0.02a	0.20±0.02a	ns	1.4±0.2^{a,b}	1.6±0.2^{a,b}	ns
Recov 72h	146±11^{b}	163±20^{c}	ns	3.9±0.1^{a,c}	3.6±0.1^{a,d}	ns	0.19±0.02a	0.18±0.03a	ns	1.3±0.3^{a,d}	1.1±0.1^{a,d}	ns

Different letters indicate statistically significant differences ($p < 0.05$) among the sampling times within each group. (*) indicates statistical differences ($p < 0.05$), whereas (ns) represents non-significant differences between the TD and TM groups. CK = creatin phosphokinase; β-HB, beta-hydroxybutyrate.

treatments across time (Table 1) and were mostly within the normal range (0.3-2.0 µg/dL; DCPAH, 2015), except for after unloading in TM calves and after 24 h recovery in TD calves, which were slightly above the normal range, reaching a mean of 2.4 µg/dL. These results agree with those of Bravo *et al*. (2018), who found non-significant increases in cortisol concentrations in calves due to 3 h transport and 24 h fasting afterwards in a pen, simulating commercial conditions. However, a higher concentration of cortisol was found in TM calves after unloading (12 h) than in animals in the same group at 72 h ($p < 0.05$), and a higher cortisol concentration at 24 h than at 72 h was found in the TD group ($p < 0.05$), indicating that there was an increase in cortisol due to transport and fasting, which tended to decrease during recovery.

The mean concentrations of β-HB were within the normal range (Wittwer, 2012) and did not differ between the treatments at any sampling time ($p > 0.05$; Table 1). Increases in β-HB concentrations indicate that animals use their body reserves due to fasting and/or exercise (Broom, 2003; Knowles *et al*., 2014). According to our results, fasting calves for 12 h, which included a total transport time of 5 h (total duration of the journeys to the market and back), plus 7 h of feed and water deprivation in the market (TM) or in a corral on the farm (TD), was not sufficient to make them use their body reserves. After 24 h of fasting (including 3 h

of transport), Bravo *et al.* (2018) found a significant increase in β-HB in calves, although the mean values were still within the normal ranges (0.1-0.6 mmol/L). It needs to be considered that the most common situation within markets is that animals remain in the premises for longer times (Bravo *et al*., 2019, 2020) than it was the case in the present study.

The average live weight of the calves was reduced by 6.5% between 0 and 12 h, without statistical differences between the TD and TM groups ($p > 0.05$, Table 2). This live weight loss is similar to the 6.8% reported earlier in calves by Bravo *et al.* (2018) after 3h of transportation and a total of 24 h of fasting in a pen without passing through a market. Live weight loss is probably the most significant economic effect associated with marketing procedures since animals are traded based on this variable (Bravo *et al*., 2019, 2020). In our study, live weight was not significantly affected by passing through the market; even when both groups were transported under the same conditions and exposed to the same fasting time, TM calves showed proportionately greater weight loss than TD calves at the final sampling (12 h) (7.5 vs. 5.7%). The initial weight before loading (0 h) recovered at 48 h in both treatments and a tendency to gain weight was observed at 72 h. It is likely that the differences in weight loss were not significant due to the short time spent in the market, the small sample size of calves, and their high weight dispersion. Therefore, experiments with more animals and less weight dispersion should be conducted, as well as replicate studies in different markets under various conditions.

Rectal temperature mean values throughout the study remained within normal rectal temperature ranges, from 38.6 to 39.4°C (University of Glasgow, 2013), but after unloading (12 h) values were higher in TM calves than in TD calves, showing that passing through the auction market increased rectal temperature ($p < 0.05$, Table 2). This result agrees with the increase in body temperature due to stress and physical exercise during marketing (King, 2004; Oka *et al*., 2001). In addition, the mean rectal temperature in TD calves was higher at 72 h than after unloading ($p < 0.05$), and in the TM group, the rectal temperature was also higher after 72 h than the initial value ($p < 0.05$). Considering that there were no calves with fever or other clinical signs of disease, this could reflect that repeated handling during daily sampling also caused some stress in the animals. An increase in tympanic temperature and maximum eye temperature associated with handling and transportation has been previously reported in beef calves transported for 3 h and fasted for 24 h without feed or water (Bravo *et al*., 2018); however, in the present study, maximum eye temperatures did not vary between treatments or sampling times (Table 2).

In a study under commercial conditions such as ours, it was difficult to standardize the different factors that could increase stress and affect the welfare of calves sold in commercial situations. Many factors could have influenced the results; therefore, few differences were found between

Table 2.
Means and standard errors (±SE) of the blood variables related to stress in only transported (TD) and transported and marketed (TM) calves at different sampling times (Initial, After Unloading (AU), and during recovery at 24, 48, and 72 h after the initial sampling).

Sampling times	Live weight (kg)			Rectal temp (°C)			Maximum Eye Temp (°C)		
	TD	TM	p	TD	TM	p	TD	TM	p
Initial 0h	176±12	173±12	ns	38.5±0.1^{a,b}	38.5±0.1^{a,b}	ns	34.4±0.3^{a}	34.3±0.3^{a}	ns
AU 12h	166±11^{a}	160±11^{a}	ns	38.4±0.1^{b,c}	38.9±0.1^{a,c}	*	34.3±0.2^{a}	34.4±0.3^{a}	ns
Recov 24h	171±11^{b}	167±12^{b}	ns	38.4±0.1^{a,b}	38.6±0.1^{a,c}	ns	33.7±0.3^{a}	33.3±0.3^{a}	ns
Recov 48h	177±12^{c}	172±12^{c}	ns	38.8±0.1^{a,b}	38.9±0.1^{a,c}	ns	34.0±0.2^{a}	34.2±0.2^{a}	ns
Recov 72h	181±12^{c}	175±13^{c}	ns	38.8±0.1^{a,d}	39.1±0.1^{c,d}	ns	33.7±0.4^{a}	34.2±0.3^{a}	ns

Different letters indicate statistically significant differences ($p < 0.05$) among the sampling times within each group. (*) indicates statistical differences ($p < 0.05$), whereas (ns) represents non-significant differences between the TD and TD groups. Letters next to the live weight values represent statistical analyses comparing the variation from the initial sampling time to other measurements in both groups. CK = creatin phosphokinase; β-HB, beta-hydroxybutyrate.

calves transported directly from farm to farm and those passing through the market. For instance, the time spent in the market by TM calves was less than the regular 12 hours previously described by Bravo *et al*. (2020). Moreover, the authors (although not measured) observed that handling by stockpersons was mostly gentle, with low stress and no rush, as there were few animals for sale. This is uncommon in Chilean livestock markets (Bravo *et al*., 2019; De Vries, 2011). This also suggests that it is possible that selling calves through the market does not significantly affect the physiological variables compared to a direct sale from farm to farm if transport and auctioning times are kept short and handling within the market is less stressful.

The limitations of this study are the small number of animals used and the absence of blood sampling of animals upon arrival at the market and after their stay on the market before loading again. Unfortunately, it is not allowed to perform any sampling or treatment within the premises; moreover, this would have meant extra handling and stress for the calves, a situation that usually does not occur within the market. Therefore, these should be considered preliminary results with a small number of calves, and experiments should be repeated with more animals in different markets and situations.

CONCLUSIONS

Contrary to expectations, we can conclude that under the conditions of our study, weaned beef calves that passed through the auction market exhibited higher final CK activity and rectal temperatures than those transported directly from farm to farm. However, these findings should be considered preliminary because of the small number of calves involved and the relatively short exposure time to the handling conditions of a livestock auction market (7 h), which are not representative of typical scenarios. Experiments should be repeated to test more animals under different conditions and handling conditions.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Ethics Statement

The Bioethics Committee "Use of animals in research" of the Universidad Austral de Chile, approved the present study (Approval N°325/2018).

Author Contributions

Conceptualization VB, CG; Data curation VB; Formal analysis VB, GAJ; Funding acquisition VB, CG; Investigation VB; Methodology VB, CG; Project administration VB, CG; Resources and software VB, CG, GAJ; Supervision CG; Validation and Visualization CG, GAJ; Writing – review and editing VB, GAJ, CG.

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SHORT COMMUNICATION

Identification of potentially harmful bacterial genera of veterinary relevance in the Llanquihue urban wetlands

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ABSTRACT. Water bodies constitute natural, social, and ecological heritage under constant threat from the footprint of human action. They are the habitats of many species and play a key role in sustaining biodiversity in different ecosystems. However, anthropogenic activity can result in eutrophication of water bodies, favoring the excessive growth of microorganisms, which can be a risk factor for animal, human, and environmental health. The Agricultural and Livestock Service [Servicio Agrícola y Ganadero (SAG)] of Chile developed a list of Mandatory Reportable Diseases (MRD) to report suspected contagious diseases of veterinary relevance. In this sense, the detection of microorganisms in water bodies for the characterization of their sanitary status is important for the development of monitoring strategies and the prevention of possible diseases. This study aimed to determine the presence of potentially harmful bacteria in Llanquihue urban wetlands. Here, we used metagenomic sequencing of amplicons to characterize the composition of microorganisms in three urban wetlands in the city of Llanquihue, which could be a risk factor for animal health. Our main finding was the detection of genera that may be associated with MRD, in addition to other microbial genera that have pathogenic potential. On the other hand, we also report the presence of cyanobacteria genera that can be involved in toxin production in water bodies as another potential source of risk. This is the first study to use massive sequencing techniques to analyze the sanitary status of urban wetlands in the city of Llanquihue.

Keywords: Mandatory reportable diseases, Wetlands, DNA sequencing, bioinformatics.

INTRODUCTION

In the Los Lagos region of southern Chile, approximately 70% of the land corresponds to hydrographic basins. Rivers, lakes, wetlands, and groundwater have sustained the most important economic, social, and productive human activities, such as agriculture, livestock, forestry, and aquaculture, among others. However, poor management of water resources, excessive exploitation, and negligence in controlling anthropogenic organic and industrial waste harm ecosystems (Zedler & Kercher, 2005). These activities promote high levels of nutrients, such as nitrogen and phosphorous, in the aquatic network, a process called eutrophication (Akinnawo, 2023). Eutrophication is one of the most worrying consequences of anthropogenic activity in ecosystems and water bodies (Bhagowati & Ahamad, 2019; Chislock *et al.*, 2013; Khan & Mohammad, 2014; Pitois *et al.*, 2001), favoring the excessive growth (blooms) of microorganisms that decrease the quality and availability of resources (Amorim & Moura, 2021; Paerl *et al.*, 2014). In addition, microorganism blooms can be a risk factor for animal health because the abundance of certain pathogens in water bodies increases the risk of infectious or toxicological diseases in wildlife and domestic animals (Parlapani *et al.*, 2023; Lapointe *et al.*, 2015; Walters *et al.*, 2011; Zinia & Kroeze, 2015).

The Agricultural and Livestock Service of Chile [Servicio Agrícola Ganadero (SAG)] has drawn up a list of Mandatory Reportable Diseases (MRD) of veterinary relevance established for reporting suspected contagious diseases in animals (DECRETO 389 EXENTO, Ministerio de Agricultura, Chile. 2014). MRD can be caused by a wide variety of pathogens belonging to different bacterial genera. Some of these bacterial genera include *Mycobacterium*, *Brucella*, and *Mycoplasma*, which are involved in diseases such as bovine tuberculosis, paratuberculosis, brucellosis, and avian mycoplasmosis, among others (SAG, 2019). However, there are other diseases that, although not currently part of the MRD list, may constitute a risk to animal health, such as species members of *Flavobacterium*, *Microcystis*, and *Leptospira* genus. In the case of *Flavobacterium*, it has been reported that the species *Flavobacterium psychrophillum* can cause flavobacteriosis disease, being the second cause of mortality in fry and salmonids in culture center (Martínez, 2018). In addition, pathogenic species of the genus *Leptospira* are involved in a zoonosis with a worldwide distribution that occurs mainly in tropical, subtropical, and temperate zones (Céspedes, 2005). In this context, mammals such as horses, cows, dogs, cats, pigs, and rodents are highly relevant in the transmission of this pathogen because they are asymptomatic carriers (Faine, 1994). However, several reports suggest that animal health is not necessarily impaired only by pathogenic microorganisms, but also by microorganisms capable of producing toxic substances or products with noxious effects (Wood, 2016).

Cyanobacteria are widely distributed oxygenic, phototrophic microorganisms. Owing to eutrophication and

the progressive increase in temperature associated with climate change, an increase in cyanobacterial blooms has been reported in marine and freshwater ecosystems worldwide (Markensten *et al*., 2010; Paerl & Huisman, 2008, 2009). One of the major concerns related to the presence of these microorganisms is the ability of some species to produce toxins (cyanotoxins) that can affect humans and animals as well as drastically decrease the health and quality of water bodies (Agrawal *et al*., 2006; Briand *et al*., 2003, 2005; Codd *et al*., 1999; Codd *et al*., 2005; Dadheech *et al*., 2001; Oberhaus *et al*., 2007). Cyanotoxins that damage the liver and/or nervous system are classified as hepatotoxins and neurotoxins (Dittmann *et al*., 2013). It has been reported that hepatotoxic and neurotoxic cyanotoxins can cause acute lethal poisoning (Roset *et al*., 2001). Microcystins are among the most common and toxic cyanotoxins in water bodies (Chorus & Welker, 2021), and toxins produced by members of the genus *Microcystis*. Microcystins can accumulate in environmentally exposed animal species, subsequently entering the food chain (Gkelis *et al*., 2006; Papadimitriou *et al*., 2010; Peng *et al*., 2010; Xie *et al*., 2005; Zhang *et al*., 2007, 2009) and affecting a wide variety of species. Therefore, the presence of microorganisms with noxious effects in water bodies is an important public health concern.

Urban wetlands are ecosystems located within an urban radius where a wide variety of animal species can interact (Jisha & Puthur, 2021). However, in very few of these ecosystems, initiatives aimed at the identification, monitoring, and risk assessment against the presence of bacterial genera that are pathogenic or pose any potential risk to other animal species have been conducted. Currently, massive sequencing tools are widely used in the study of microbial communities in different environments and allow, among other things, the detection of pathogens (Bass *et al*., 2023). In this study, we used amplicon sequencing to describe the composition of microorganisms in three urban wetlands in the city of Llanquihue, which may represent a risk factor for animal health.

MATERIAL AND METHODS

Samples

Water samples were collected from three wetlands in the city of Llanquihue (Figure 1), located in northern Patagonia, southern Chile. A volume of 2 liters of water samples was collected from the wetlands El Loto (41°15'16.4 "S 73°00'32.9 "W), Baquedano (41°15'01.2 "S 73°00'31.9 "W), and Las Ranas (41°15'43.4 "S 73°00'24.1 "W) in two different points of each wetland, using sterile glass bottles. The samples were recovered on May 5, 2021. The samples were transported using ice packs at 4°C for processing at the Institutional Laboratory of the Universidad San Sebastián, located in the city of Puerto Montt. The water was passed through MCE (Mixed Cellulose Ester) filters of 0.22 µm pore size, using a vacuum filtration system. The filters with biological material were stored in RNA Later (SIGMA, USA) at -20 °C until nucleic acid extraction.

Genomic DNA extraction

To recover gDNA, the filters were suspended in TRIS-EDTA-NaCl (STE) buffer at concentrations of 200 mM TRIS, 200 mM NaCl, and 20 mM EDTA prepared in DNAse-free water. Enzymatic digestion of the cell wall was performed using 20 µL lysozyme (20 mg/mL) and 20 µL proteinase K (20 mg/mL), incubated for 1 h at 37 °C, and then the temperature was raised to 55 °C for an additional hour. During incubation, the solution was vortexed to allow the biological material of microorganisms to be released from the filters. The obtained solution was used for gDNA extraction using affinity columns provided in the AccuPrep® Genomic DNA Extraction kit (BIONEER, Korea), following the manufacturer's instructions.

DNA Amplicon Sequencing

A minimum of 200 ng of total DNA was sent to Novogene Sequencing Service (USA) for DNA amplicon sequencing of the 16S rRNA taxonomic marker. For this, the V3-V4 region (F: CCTAYGGGRBGCASCAG, R: GGACTACNNGGGTATCTAAT) was amplified under previously established conditions (Behrendt *et al*., 2011). The amplicons obtained were sequenced using Illumina Novaseq 6000 equipment. Sequencing was performed using paired ends with a length of 150 bp (150 bp paired-end) and an output of 3 gigabases per sample. Sequences were delivered in FASTQ format.

Bioinformatic Analysis

The obtained sequences were imported into the R language version 4.1.0 (R Core Team, 2013) to be processed following the DADA2 v1.16 pipeline (Callahan *et al*., 2016) using the RStudio environment (Booth *et al*., 2018). The preprocessing performed included inspecting quality profiles, filtering, and trimming of low-quality data. The obtained files were used to learn the error rates and generate an error model based on the data structure. Subsequently, the information obtained was combined and a sequence table was constructed to remove chimeric artifacts resulting from PCR amplification.

Taxonomic assignment was performed using the SILVA SSU V138 database (Quast *et al*., 2013). The non-rarefied information was used to construct a phyloseq object (McMurdie & Holmes, 2013) to estimate taxonomic abundance and microbial diversity. The Microbiome package was used to estimate the core diversity (Lahti *et al*., 2017). Finally, all data were plotted using the ggplot2 library (Ginestet, 2011) following the instructions of the phyloseq package (McMurdie & Holmes, 2013). Relative abundance was plotted using the average of the replicates from each site.

RESULTS AND DISCUSSION

Microbial diversity of urban wetlands from Llanquihue City

Using 16S rRNA amplicon sequencing of water samples from the El Loto, Las Ranas, and Baquedano wetlands, we performed taxonomic assignment of microbes that inhab-

it these ecosystems. After describing the high diversity of microorganisms at the phylum level, sequencing data were separated into independent sets for each wetland studied (Figure 1A). The relative abundance at the phylum level was estimated for each wetland (Figure 1B). The results indicated a heterogeneous representation of the diversity of microorganisms present in the three water bodies considered in this study, with Las Ranas being the wetland with the highest diversity (Figure 1C). It is worth noting the high abundance of cyanobacteria in the El Loto wetland, which was considerably higher than that in the Baquedano and Las Ranas wetlands. Additionally, other phyla including potentially pathogenic species such as *Actinobacteriota*, *Spirochaetota*, and *Bacteroidota* were observed.

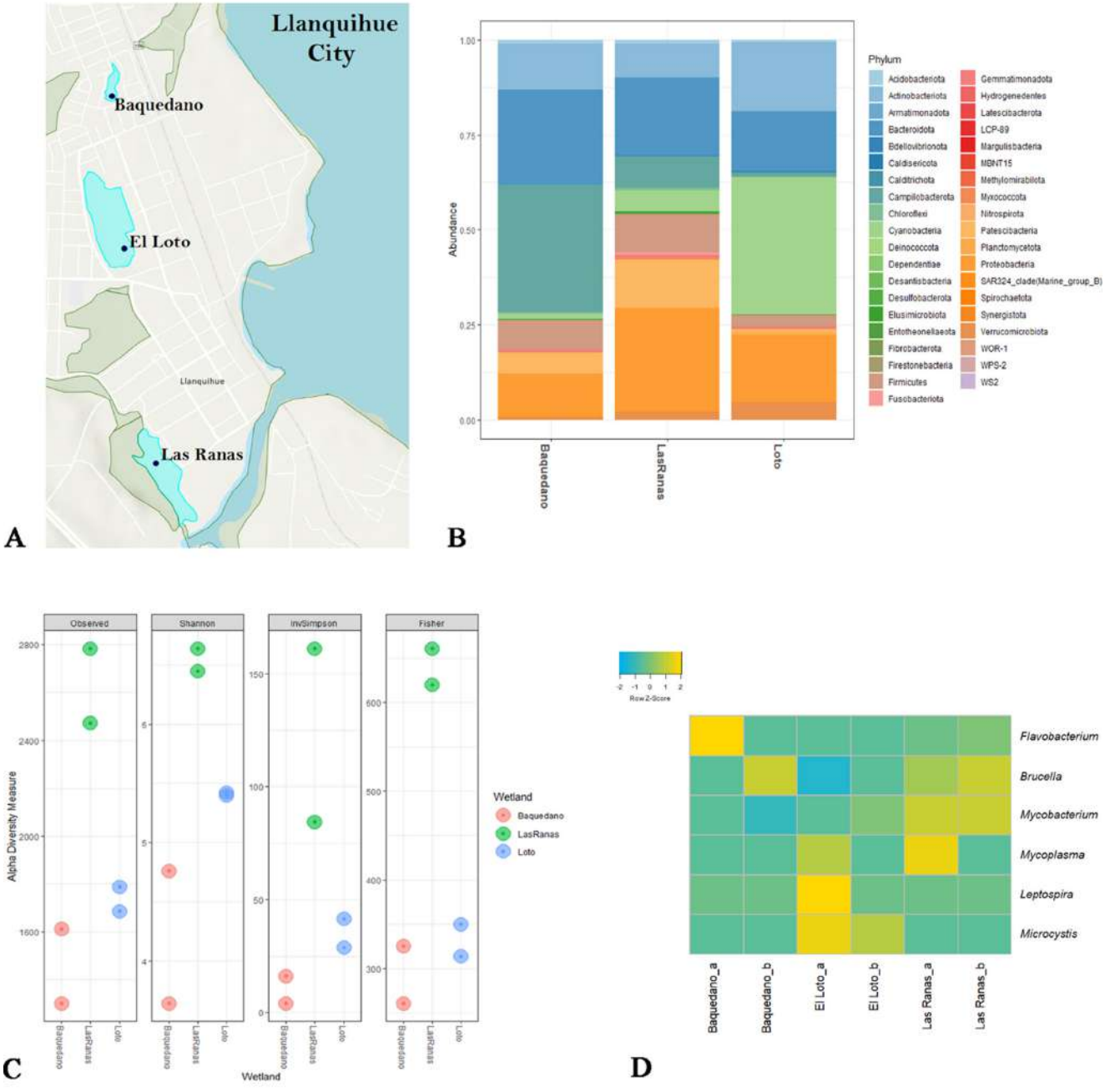

Figure 1.
A) Three Urban wetlands located in Llanquihue city were selected for microbiological description. Baquedano wetland (1, 41°15'01.2"S 73°00'31.9"W), El Loto wetland (2, 41°15'16.4"S 73°00'32.9"W), Las Ranas wetland (3, 41°15'43.4"S 73°00'24.1"W). B) Relative abundance at phylum level obtained from urban wetlands. C) Measure of alpha diversity. D) Z-score of relative abundance of genera that may be associated with MDR or with harmful species.

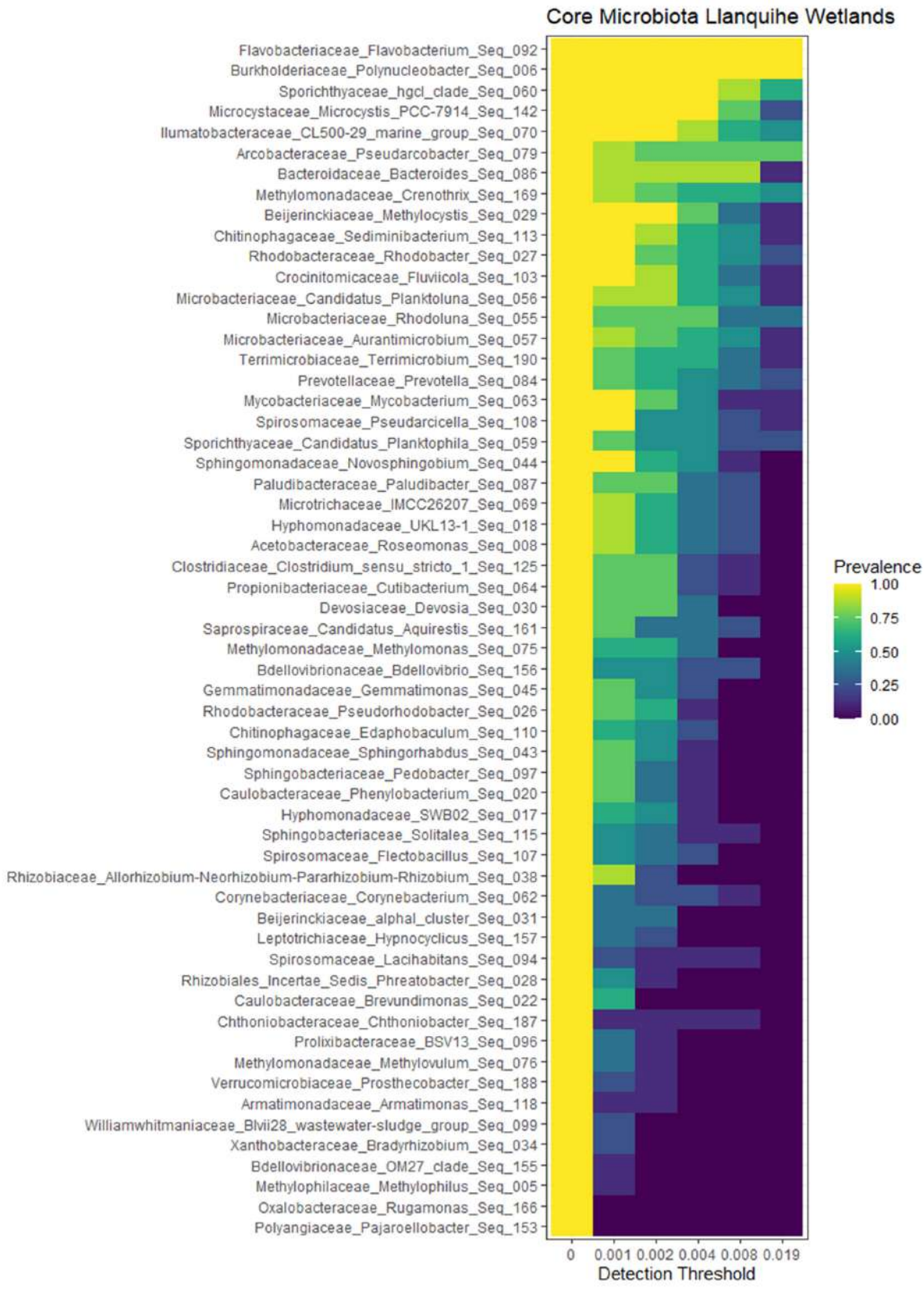

Figure 2.
Core diversity analysis of the three urban wetlands. Taxonomic abundance data obtained from the wetlands were used to estimate the core of the most prevalent microorganisms inhabiting wetlands. The detected genera are ordered along the Y-axis

When examining the taxonomic classification at lower levels, we found the presence of genera that possess species with pathogenic potential, which are even present in the list of MRD. Figure 1D shows the presence of these genera in the three wetlands: *Mycobacterium*, *Brucella*, *Mycoplasma*, *Leptospira*, *Flavobacterium*, and *Microcystis*. It is well known that a variety of species belonging to these genera have serious implications on health (for reviews, please see Bierque *et al*., 2020; Byndloss & Tsolis, 2016; Dow & Alvarez, 2022; Evangelista & Coburn, 2010; Garvey, 2020; Hilborn & Beasley, 2015; Irshath *et al*., 2023; Loch & Faisal, 2015; Olea-Popelka *et al*., 2017; Olsen & Palmer, 2014). Taken together, the presence of these bacterial genera corresponds to the first warning signal for the development of preventive measures.

Analysis of the Core diversity of the microbiota

Using the relative taxonomic abundance obtained, an analysis was carried out to identify the microorganisms that make up the microbial core, which consisted of the most prevalent shared microorganisms between niches (Shetty *et al*., 2017) in the three Llanquihue wetlands analyzed (Figure 2). The results indicate that cyanobacteria belonging to the taxa *Flavobacterium*, *Polynucleobacter*, *Sporichthyaceae*, and *Microcystis* are the four top bacterial groups, which include species of health concern, such as *Flavobacterium* (Nematollahi *et al*., 2003) and *Microcystis* (Carmichael, 1996). Interestingly, wetlands include genera that own species with pathogenic potential, such as *Roseomonas* and *Clostridium* (Mitchell *et al*., 2022; Loch & Faisal, 2015; Nematollahi *et al*., 2003). In addition, the genus *Microcystis* has toxicological potential (Carmichael, 1996).

The high abundance of genera with sanitary relevance, such as *Microcystis* and *Flavobacterium*, suggests that these water bodies can be reservoirs and dispersion media for MDRs and noxious species. In the case of *Microcystis* genera, there are few reports in Chile about the occurrence of a toxic bloom of *Microcystis aeruginosa* in lakes of the Biobío region, demonstrating that blooms occur periodically throughout the time, forming cumulative blooms during the summer; during the rest of the year, they can form a dispersive bloom (Almanza *et al*., 2016). Additionally, Nimptsch *et al.* (2016) detected toxins associated with cyanobacterial blooms in lakes in northern Chilean Patagonia. However, no reports are available on the urban wetlands in the Los Lagos region. In addition, there is no database in this region with reports of animal toxicosis or human illnesses associated with ingestion of toxin-producing cyanobacteria. Nevertheless, it is necessary to develop preventive monitoring strategies for potentially toxic cyanobacterial species, as this phenomenon is on the rise worldwide and its impact on health, the environment, and the economy can be harmful, mainly because the management of *Microcystis* blooms is complex (Wilhelm *et al*., 2020).

It is advisable to take preventive rather than reactionary action. In the microbial core, we only found the presence of *Bacteroides* and *Prevotella* (Figure 2). We detected the presence of genera whose species are described as belonging to intestinal microbiota, such as *Prevotella* (Tett *et al*., 2021), *Coprococcus* (Holdeman & Moore, 1974), *Bifidobacterium* (Bunesova *et al*., 2014), and *Ruminococcus* (La Reau & Suen, 2018), which are in concordance with the fecal presence in the studied urban wetlands (data not shown).

Currently, the One Health perspective for research that unifies animal, human, and environmental health, requires multidisciplinary approaches to deepen the understanding of microbial communities that inhabit a given environment (Oliveira *et al*., 2023; Banerjee & van der Heijden, 2023; Zinsstag *et al*., 2018; Farschtschi *et al*., 2022; Hilborn & Beasley, 2015). An increasing amount of evidence suggests that experimental strategies including global analyses of DNA, RNA, metabolites, and proteins (areas of research classified as "omics") promising alternatives to conducting One Health studies (Gruszecka-Kosowska *et al*., 2022; Tigistu-Sahle *et al*., 2023) and the possibility to assess these studios in a variety of environments, ecosystems or conditions. Nevertheless, the presence of genera associated with MRD or noxious species raises the possibility that the same event may occur in other cities located around water bodies, which could affect the health of its inhabitants. Fortunately, the rise of molecular and DNA sequencing tools offers a new range of possibilities for the development of management strategies and monitoring of environmental microbial threats.

Further research is required to identify which species associated with MRD can be found in other water bodies in the region. It is necessary to implement techniques that allow characterization of the presence of microorganisms with sufficient resolution to determine the species inhabiting these ecosystems. Because water bodies are complex environments with a variety of microbial communities inhabiting them, Shotgun Metagenomics Sequencing may be an alternative to carry out deeper microbial and functional characterization of environmental communities. These methodologies are used for the massive detection of pathogen genomes in different environments and enable the study of interactions among human, animal, and environmental microbiomes (Trinh *et al.* 2018).

Authors' contributions

JDR wrote the manuscript and discussed results. DAM directed the study, conceptualized the experiments, performed bioinformatics analyses, and edited the manuscript. CO performed the experiments and conceptualized the figures. VC and CM contributed to reviewing the manuscript and discussed results. All authors agree with the final version of the manuscript.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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SHORT COMMUNICATION

Experimental assessment of the efficacy of copper ion treatment against penicillin G contained in UHT milk and PBS

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ABSTRACT. Antibiotics are widely used in animal production to treat bacterial infections and to improve performance and animal welfare. Their misuse poses a threat to public and animal health because of the possible development of antibiotic-resistant microorganisms. Among the many strategies that have been considered to address this problem are methods to degrade antibiotic residues, especially those from the human and animal food chain. This study describes the effect of copper ion treatment on the detection of penicillin G in a liquid matrix. An *in vitro* experimental study was designed using both commercial milk and PBS spiked with three different concentrations of penicillin G. Each sample was treated for 30 min with copper ions. All samples were tested for antibiotics before and after treatment using a commercial enzyme-linked receptor binding assay. Additionally, pH, copper concentration, and temperature were evaluated. Antibiotic residues were detected in all spiked PBS and milk samples before treatment with copper ions. However, after 30 min of treatment, no antibiotic residues were detected in any sample at any concentration tested. In conclusion, treatment of penicillin-contaminated milk and PBS samples with copper ions affects antibiotic detection, which would potentially reduce antibiotic levels.

Keywords: antibiotics, copper ion treatment, penicillin G, milk, PBS.

INTRODUCTION

Antibiotics are widely used in animal production to treat bacterial infections and to improve animal performance. Although antibiotics play an important role in reducing the mortality rate, increasing production, and improving animal welfare, their misuse has raised concerns about public health and food safety owing to the potential development of antibiotic-resistant microorganisms (Ricci *et al*., 2017).

Intramammary infections are one of the most frequent bacterial infectious diseases in dairy cows and are the main reason for antibiotic use in adult cattle (Stevens *et al*., 2016). Waste milk (WM) is a term used to describe milk from cows with intramammary infections treated with antibiotics but may also refer to milk contaminated with other drugs, milk from cows with clinical mastitis, milk with high somatic cell counts (SCC), and post-colostrum transition milk (Ricci *et al*., 2017).

Although the use of WM in calf feed is controversial because of its role in pathogen transmission (Selim & Cullor, 1997) and the risk of selection for antibiotic-resistant bacteria (Ricci *et al*., 2017), it is widely used by dairy farmers (Calderon-Amor & Gallo, 2020).

The spread of antibiotic-resistant bacteria to the environment and their subsequent transmission to humans are considered one of the main threats to global public health (WHO, 2014).

Therefore, several methods have been evaluated to degrade antibiotic residues in WM, such as incubation with β-lactamases, electrochemical oxidation, heat, or pH treatment. However, none of these methods have been applied in the field (Ricci *et al*., 2017).

It has been demonstrated that the complexation of different metal ions can affect the properties and stability of β-lactam antibiotics (Deshpande *et al*., 2004). Among the metal ions, copper in the ionic form (Cu^{II}) has a greater catalytic effect on the degradation of penicillins than Zn^{II}, Ni^{II}, or Co^{II} (Gensmantel *et al*., 1980). The degradation of β-lactams by Cu^{II} can occur via hydrolysis, oxidation, or both, depending on the type of β-lactam. For example, the degradation of penicillin G in water occurs via hydrolysis, followed by oxidation of the products of this hydrolysis (Chen *et al*., 2016). Under mildly acidic conditions, both penicillin G and V were hydrolyzed by the cupric ion into penicilloic acids (Niebergall *et al*., 1966).

Recently, our research group evaluated an antibacterial principle based on copper ions for decontaminating bovine milk. This principle was capable of significantly decreasing the load of viable pathogens such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *S. aureus*, and *E. coli* in an *in vitro* study (Steuer *et al*., 2018).

Taking all these considerations into account, the present study aimed to evaluate the effect of copper ions on

the detection of penicillin G in milk by adding three concentrations of penicillin G sodium to ultra-high-temperature (UHT) milk and PBS.

MATERIAL AND METHODS

Experimental design

To fulfil the proposed aim, an *in vitro* experimental study was conducted. Phosphate saline buffer (PBS) and UHT commercial milk were used as liquid matrices. Volumes of 500 mL were taken from each matrix, into which potassium penicillin G (Merck KGaA, Darmstadt, Germany) was added to reach concentrations of 20, 200, and 1,000 ppb. Each sample was treated for 30 min with a copper treatment device consisting of a glass receptacle, in which two high-purity copper plates were immersed. The copper plates were stimulated with a low-voltage (24 V) electric current (3 A) to quickly release copper ions (Steuer *et al.*, 2018). PBS and milk samples without antibiotics were used as negative controls. In addition, pH controls were added using PBS and milk contaminated with 1,000 ppb penicillin and incubated for 30 min at pH 11.5. All samples were tested for antibiotic detection before and after treatment with the commercial enzyme-linked receptor-binding assay IDEXX SNAPduo ST Plus Test (Idexx Laboratories Inc., Westbrook, ME, USA). The pH, Cu concentration, and temperature of the samples were also evaluated.

Statistical analysis

All analyses were performed using R Statistical Software (v4.2.1; R Core Team 2022) and Excel 2016 (Microsoft Corp., Redmond, WA). The Wilcoxon signed-rank test was used to determine significant differences in each response variable (Cu concentration, pH, and temperature) before and after Cu treatment. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

In all PBS and milk samples where the three concentrations of penicillin (20, 200, and 1,000 ppb) were added, antibiotic residues were detected before treatment with copper ions. However, after 30 min of treatment, no antibiotic residues were detected in any of the samples (Figure 1).

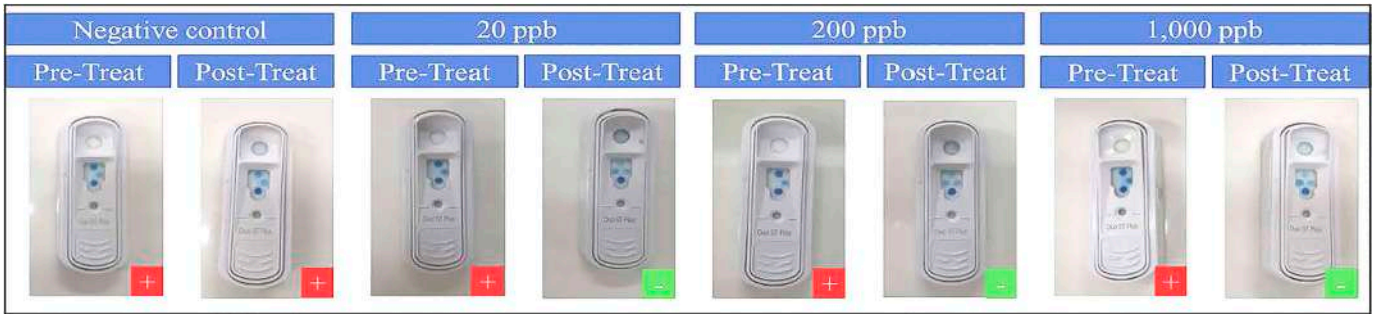

Figure 1. β-Lactam detection in milk with SNAPduo ST Plus Test before and after 30 min treatment with copper ions. The blue dot on the left side of the reading window indicates no detection of β-lactam antibiotics in the sample (–). If the blue dot was missing, the sample contained a β-lactam antibiotic residue (+).

Interestingly, when samples were incubated at an alkaline pH as a treatment, antibiotic residues were detected before and after treatment.

In the PBS, the average pH was 8.06 (SD 0.06) in pretreated samples and 11.74 (SD 0.37) in post-treated samples ($p < 0.05$). The average copper concentration was 0.03 mg/L (SD 0.03) in pretreated samples, and 525.91 mg/L (SD 07.39) in post-treated samples ($p < 0.01$). The average temperature was 20.80 °C (SD 1.28) in the pretreated samples and 27.53 °C (SD 1.36) in the post-treated samples ($p < 0.05$) (Table 1).

In milk samples, the average pH was 6.58 (SD 0.03) in pretreated samples and 9.10 (SD 0.159) in post-treated samples ($p < 0.05$). The average copper concentration was 0.40 mg/L (SD 0.25) in the pretreated samples and 1,698.82 mg/L (SD 162.06) in the post-treated samples ($p < 0.01$). The average temperature was 19.98 °C (SD 1.04) in pretreated samples and 33.98 °C (SD 1.24) in post-treated samples ($p < 0.05$) (Table 1).

For dairy farmers, WM implies economic loss because it cannot be marketed for human consumption. This loss has been estimated to range from US$3.7 to US$23 per cow per year (Hogeveen *et al.*, 2011). However, most concerns relate to the effects of its use as calf feed on the selection and transmission of antibiotic-resistant bacteria (Ricci *et al.*, 2017).

In this study, milk and PBS samples were contaminated with penicillin G to assess the effect of copper ion treatment on residue detection. Penicillin is one of the most common antibiotic residues detected in WM (Selim & Cullor, 1999; Pereira *et al.*, 2014a). Furthermore, it is commonly used in studies as an antibiotic model to

simulate WM (Pereira *et al*., 2014b; Pereira *et al*., 2016). The three concentrations of penicillin used in the present study were chosen based on the range of concentrations found in WM (Pereira *et al*., 2014a) and the detection limit of the SNAP test (2 ppb). The sensitivity of the test used for the penicillin, cephalosporin, and tetracycline groups was 4, 30, and 18 ppb, respectively. The SNAP test detects beta-lactam antibiotics, cephalosporins, and tetracyclines by binding to enzyme-linked receptors.

Among the methods that have been described for degrading antibiotic residues contained in WM, heat treatment, pH treatment, and electrochemical methods can be highlighted. Heat treatment is widely used as a method to reduce bacterial populations in WM, but it can also reduce the concentrations of certain antibiotics. Heat treatment of milk at 120 °C for 20 min degraded 47% amoxicillin, 84% ampicillin, 53% cloxacillin, and 61% penicillin G (Roca *et al*., 2011). Recently, Garzon *et al*. (2020) reported that heating milk at 92 °C for 20 min could degrade 35.24% of the initial concentration of ceftiofur. In the same study, the authors described that alkalinizing milk to pH 10 resulted in a 95.72% degradation of the initial concentration of ceftiofur. In the present study, one effect of copper ion treatment was to increase the pH of all milk and PBS samples to 11.74 and 9.10, respectively. The latter is complemented by what was published in Božić *et al*. (2018), where it was established that penicillin G did not show any coordinative or redox interaction with $Cu2^{+}$ in phosphate buffer at a physiological pH of 7.4.

However, in the samples spiked with 1,000 ppb of penicillin and alkalinized to pH 11.5, β-lactam residues could be detected before and after treatment, showing no effect of pH on residue detection. This difference could be explained by the difference in the structure of the antibiotics (ceftiofur – penicillin) and detection methods (HPLC-SNAP).

In experimental trials, there is evidence of the efficacy of electrochemical oxidation of metals for the degradation of antibiotics in waste milk. Kitazono *et al*. (2017) demonstrated the degradation of chlortetracycline and cephalozine with ions produced by the electrical stimulation of a titanium cathode, while Elmola & Chaudhuri (2009) demonstrated the degradation of amoxicillin, ampicillin, and cloxacillin using the Fenton reaction, which involves the use of iron ions and hydrogen peroxide. Electrochemical oxidation is a process in which organic substances are oxidized and converted into nontoxic substances under the action of an electric current (Wang & Zhuan, 2020). Kitazono *et al*. (2012), who found an 83% reduction in the concentration of oxytetracycline after a 6-hour treatment, used electrochemical oxidation of metals for the degradation of antibiotics in WM. In a later study, Kitazono *et al*. (2017) demonstrated the degradation of chlortetracycline and cephalozine with ions produced by electrical stimulation of a titanium cathode. The degradation of antibiotics in other aqueous matrices in the presence of various metal compounds has also been demonstrated (Wang & Zhuan, 2020).

A study by Riediker *et al*. (2004) showed that milk with penicillin G, amoxicillin, and ampicillin stored for six days at 4 °C experienced a degradation of more than 50%. However, no degradation was observed in milk spiked with ceftiofur and stored for 14 d at 4 °C (Karageorgou & Samanidou, 2010).

The widespread use of WM in calf feeding represents a challenge in the development of efficient methods for antibiotic removal. The results of this study suggest that electrochemical oxidation with copper ions affects the detection of penicillin in milk and could potentially be a method for degrading penicillin G residues in waste milk to avoid environmental contamination. If this treatment is confirmed to be effective for treating waste milk, we should monitor the possible effect of copper on the health of calves. Steuer et al (2021), reported no evidence of copper toxicity after the application of copper ion to control MAP in milk intended to feed calves, based on the plasma activity of the liver enzymes evaluated, and hepatic copper concentrations were also normal. However, the Cu concentration in milk after treatment was significantly lower than that reported in the present study.

Further trials with other antibiotic detection techniques and evaluations of their post-treatment biological activities are needed.

Competing interests statement

The authors declare that they have no conflicts of interest.

Ethics statement

No experimental animals or animals for clinical research were used in this study

Author contributions

FU and MS conceived the ideas of the study. FU, MV, and CT performed laboratory assays. FU and MS led the writing of the manuscript. MS obtained the funding for this study. All the authors revised the manuscript, critically contributed to the drafts, and approved the final version for publication.

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