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Assessment of antibacterial effect of different extracts of Thymus Vulgaris against Clostridium Perfringens in chicken

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ABSTRACT

Necrotic enteritis in chicken is caused by the *Clostridium perfringens*, which have been found on almost chicken and for decades antimicrobial therapy was the only strategy to control it. However, the misuse of antimicrobials linked to many impacts like reduced susceptibility of *Cl. perfringens* strains and cause cross-resistance to antimicrobial agents. This work demonstrated that, the uses of herbal extracts with antibacterial effects consider as an effective way to prevent and reduce the Necrotic enteritis. The present study determines antimicrobial activities of methanolic, ethanolic and watery extracts of *thymus vulguaris* against *Clostridium Perfringens* type A in chicken. The antibacterial activity was assessed through measuring sensitivity of isolated strains to different extracts by agar diffusion technique and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by using the broth macrodilution method. *Thymus vulgaris* different extracts consider as effective anticlostridial herbal agent. The result of agar well diffusion assay and broth macrodilution method showed that, the tested strains were more sensitive to the ethanolic and methanolic extracts which give the lowest (MIC) and (MBC) (32, 16 respectively) than the watery extracts (512, 256 respectively).

1. INTRODUCTION

Acritical enteric disease in chicken is Necrotic enteritis which cause economic profitability losses which caused by *Clostridium perfringens* (Emami et al., 2019). Necrotic enteritis characterized by diarrhea and high mortality rate (Kumar et al., 2019). Clostridium perfringens is a soil-borne microorganism found in poultry farms between faeces, feed and on the floor (Ficken and Wages, 1997). It is a Gram-positive bacilli, anaerobic microorganism, generally produce five toxins (A to E) (Songer1996; Petitetal.1999). Clostridium perfringens type A causes necrotic enteritis in poultry which produces the chromosomal encoded alpha toxin (Songer 1996; Engstrom et al. 2003) for deep years, antimicrobial used to be the first, and almost concerning the instances the only strategy to prevent *Clostridium perfringens* (Diarraand Malouin, 2014) while to limit the necrotic enteritis occurrence, antimicrobial agents are used at sub inhibitory doses for long periods in feed (Olazabal et al., 2005). Antimicrobial growth promotors which used in feed depend on different factors as decrease susceptibility of poultry *Clostridium perfringens* strains and may cause cross-resistance to antimicrobials (Redondo et al., 2015). The uses of herbal extracts with antibacterial impact shows up as a promising and effective in controlling of necrotic enteritis (Carrasco et al., 2016). Medicinal plants are utilized in animal feed, for their antibacterial and antioxidant effects (Hashemi et al., 2010; Vondruskova et al., 2010). Thyme oil is a commercial essential oil, it is used as a food additive, has antioxidant, antibacterial, and antifungal impacts (Rasooli et al., 2006; Ben-Jabeuret al.,2015). The antibacterial effect of the *Thyme oil* adverse the most of resistant strains is observed widely (Nabavi et al.2015). So, we aimed in this study to evaluate the effect of (*thymus vulgaris*) plant extract on *Cl. perfringens* invitro by using different forms of thymus vulgaris extract on different strains of *Cl. Perfringens*

2. MATERIAL AND METHODS

2.1. Source of samples:

Total number of 123 intestinal samples have been collected from chicken from different labs and farms in Al Sharkia and Al Dakahlia Governorates during the period from October 2018 to December 2018. Samples were isolated from infected chickens with history of brownish diarrhea, high mortality rate and postmortem lesion of necrotic enteritis and apparent healthy birds. This data was shown in Table (1).

2.2. Isolations and identifications of Cl. perfringens:

Inoculate samples into cooked meat media incubated anaerobically at 37°C for 24-48hrs. Take a loopful from it and cultivated on neomycin sulphate blood agar

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anaerobically. Then examined the colonies microscopically (Collee et al., 1996).

2.3 . Extraction of DNA and PCR assay:
Extract DNA from pure colonies of *Cl. perfringens* that appeared as double inhibition zone of hemolysis by using an extraction kit. Table (2). Multiplex PCR was applied. (van Asten et al., 2009). Analyze PCR result by agar gel electrophoresis (Ghoneim and Hamza, 2017).

2.4. Antimicrobial sensitivity test using agar disk-diffusion method
Inoculate the standard suspension of the isolated microorganism in agar plates. Then, use filter paper discs, containing the extract with known concentrations from supernatant, are placed on the plate and incubated anaerobically. The antibacterial agent diffuses into the agar and inhibit the growth of the tested microorganism, then measuring of the inhibition zone (Jorgensen and Ferraro, 2009).

2.5. Assessment of antibacterial activity of methanol, ethanol, watery extracts of thymus vulgaris against Cl. perfringens
2.5.1. Preparation of Thymus vulgaris Different extraction:
Plant was purchased in leaves from Agricultural Research Institute of vegetables and horticulture, Dokki in April (Spring) then extracted in the Pharmacology Department, Faculty of veterinary medicine, Zagazig University. Thyme leaves were dried then grounded and stored until use.
To prepare the watery extract, use (100) grams of thyme powder, put in the flask containing (500) cm^3 of distilled water, mixed well for (30) minutes and centrifugated for (15) minutes. Then put the prepared solution in the electric furnace at (35) °C until we obtain the extract (Ba - Angood et al., 1996).
Alcoholic extract is prepared by putting (50) g of thyme powder in an extraction unit (Soxhelt) and added (350) ml of ethanol (80%) and use (100) g of thyme powder with (500) ml of methanol 80% for (12) hrs at temperature of (40) °C (Harborne, 1973).
2.5.2. Determination of antibacterial activities of Thymus vulgaris by agar well diffusion method:
The same procedures are used in agar well diffusion technique, inoculate the microbial suspension on the agar plate surface. Then, bores with 6 to 8 mm diameter is made by a sterile borer, and put (100 µL) of three thyme different extracts (methanolic, ethanolic and watery) at concentration of (25%, 50%, 100%) were put in the wells and incubated anaerobically (Balouiri et al., 2016).

2.5.3. Determination of (MIC) and (MBC) using broth macrodilution method:
The procedure was preparing two-fold dilutions of the antimicrobial agent (100µg/mL) in 2 mL of liquid media. Each tube is inoculated with a microbial isolate prepared in the same medium after preparing of suspension with 0.5 McFarland scale=$1.5*10^8$ then the tubes were incubated under anaerobic condition (CLSI, 2012).
MBC and MIC are detected by broth microdilution and sub-cultivation of a sample from tubes. Determine the number of colonies (CFU/mL) after 24 h of incubation anaerobically.

Table 1 Number of intestinal samples collected from different farms and labs in Sharkia and Al Dakahlia Governorates

Source of sample	Governorates	Number of intestinal samples	State of health	
			Infected	Suspected/appearance healthy
El attar farm	Dakahlia	20	11	9
El azzazi lab	Sharkia	51	28	23
Zagazig research center reference lab	Sharkia	14	9	5
Dr. Mohamed Agha Consulting Center for Poultry	Dakahlia	9	4	5
Al rowad Lab	Dakahlia	29	17	12
Total		123	69	54

Table 2 The primers nucleotide sequences used in this study

Primer	Sequences 5'---------3'	Amplified product	Reference
CpA(alpha toxin)	F-GTTGATAGCGCAGGACATGTTAAG	402 bp	Yoo*et al*., 1997
	R-CATGTAGTCATCTGTTCCAGCATC		
CpB(beta toxin)	F-ACTATACAGACAGATCATTCAACC	236 bp	
	R-TTAGGAGCAGTTAGAACTACAGAC		
CpeE(epsilon toxin)	F-ACTGCAACTACTACTCATACTGTG	541 bp	
	R-CTGGTGCCTTAATAGAAAGACTCC		
CpI(iota toxin)	F-GCGATGAAAAGCCTACACCACTAC	317 bp	
	R-GGTATATCCTCCACGCATATAGTC		

3. RESULTS

3.1. Incidence and toxin typing of *Cl. Perfringens* recovered from chicken
This work shows that, the incidence of *Clostridium perfringens* isolated from chicken intestinal samples from different farms and labs was found to be 28/123(22.7 %) (All *Cl. perfringens* which isolated were defined as type A toxin (Fig.1).

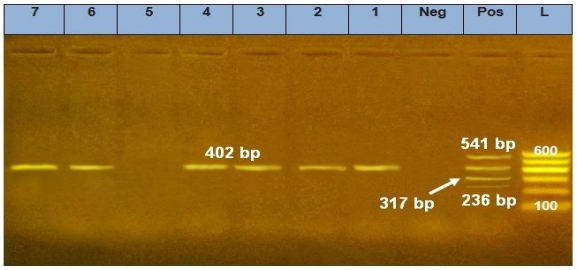

Fig. 1 Multiplex PCR of C. perfringens obtained from intestinal content Lanes 1-4 and 6-7 C. perfringens type A which amplified at 402bp. Lane 5: negative

3.2. Antimicrobial activity of *methanol, ethanol and watery extracts of thymus vulgaris on Cl. perfringens:*

Different extracts of *Thymus vulgaris* showed anti-clostridial activity against the two strains which obtained from isolated samples and reference strain obtained from Animal Health Research Institute, Dokki. Results of the agar well diffusion method assays and measurement of MIC and MBC confirmed that the tested strains were more sensitive to ethanol and methanol extracts which give the lowest MIC and MBC values (32, 16 respectively) than the watery extracts (512, 256 respectively). (Table 3 and 4).

Table 3 Inhibition zone diameters of *thymus vulgaris* Methanol, Ethanol and Watery extracts against *Cl. Perfringens* by using well diffusion method:

Extract Concentration (mg/ml)	Control	Reference Strain**	Strain1***			Strain2***		
			area	difference	Percent difference	area	difference	Percent difference
Methanol extract								
25.0%	0	2.2	1.4	-0.8	^44.4	2.1	-0.1	^4.7
50.0%	0	2.6	1.7	-0.9	^41.9	2.5	-0.1	^3.9
100.0%	0	**2.6**	**3**	0.4	*14.3	**3**	0.4	*14.3
Ethanol extract				.	.		.	.
25.0%	0	2	1.5	-0.5	^28.6	1.7	-0.3	^16.2
50.0%	0	2.4	2	-0.4	^18.2	2.1	-0.3	^13.3
100.0%	0	2.6	2.6	.00	.00	3	0.4	*14.3
Water extract				.	.		.	.
25.0%	0	2	1.5	-0.5	^28.6	1.5	-0.5	^28.6
50.0%	0	2.2	1.9	-0.3	^14.6	2	-0.2	^9.5
100.0%	0	2.4	2	-0.4	^18.2	2.1	-0.3	^13.3

^ less than reference diameters. * more than reference diameters. **ATCC®13124™*C.perfringens* reference stain. *** Strain 1, 2: strains of *Cl. perfringens* type A

Table 4 The Minimum Inhibitory Concentration of *thymus vulgaris* extracts against *Cl. perfringens* by using broth macrodilution method:

thymus extract (µg/ml)	Reference strain		Strain1		Strain2	
	MBC	MIC	MBC	MIC	MBC	MIC
Methanol	64	128	16	32	16	32
Ethanol	16	32	8	16	16	32
Water extract	256	512	256.	512	128	256

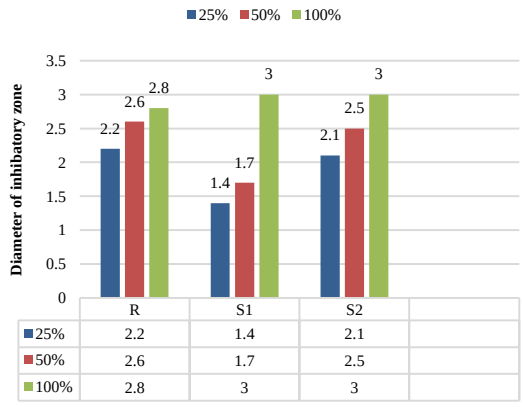

Fig. 3 Antimicrobial effect of ethanolic extract of thymus vulgaris in different concentration(25%-50%-100%) on 3different strains .

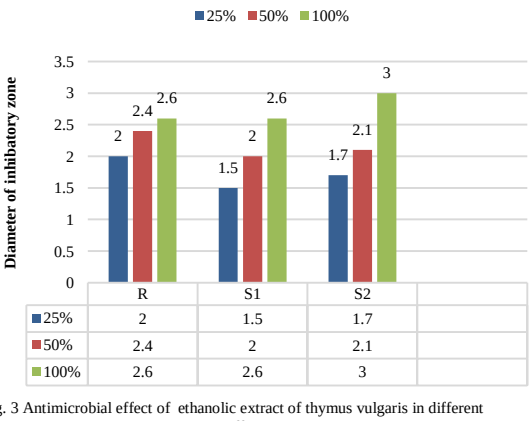

Fig. 2 Antimicrobial effect of methanolic extract of thymus vulgaris in different concentration(25%-50%-100%) on 3different strains .

Fig. 4 Antimicrobial effect of watery extract of thymus vulgaris in different concentration(25%-50%-100%) on 3different strains

4. DISCUSSION

Necrotic enteritis is caused by the bacterium *Clostridium perfringens*, a soil-borne organism found on almost every poultry farm in dust, faeces, feed, poultry litter and intestinal contents, as well as in the soil (Ficken and Wages, 1997). Clostridium perfringens is the aetiological agent of a wide range of diseases in humans and animals. Necrotic enteritis, one of the most economically important and financially crippling enteric poultry diseases in broiler chickens, causes the more commonly recognized fulminant infection which can result in outbreaks with mortality rates of up to 50% (McDevitt et al., 2006). Recent studies have shown most *Cl. perfringens* strains isolated from necrotic enteritis outbreaks are resistant to some of commonly used antibiotics such as gentamicin and streptomycin (Park et al., 2015). In this context, the utilization of natural plant extracts with antimicrobial properties appears as a promising and feasible tool to control necrotic enteritis in chicken (Crasco et al., 2016)

Concerning the antibacterial activity of methanol extract, ethanol extract, watery extract of *thymus vulgaris* against *Cl. perfringens*; the results from the agar well diffusion method assays showed variable activity against all tested

strains of *Cl. perfringens.* The highest susceptibility was recorded for strain 1 and 2 against 100% methanolic extract and for strain 2 against 100% ethanolic extract. Thymus vulgaris different extracts exhibited considerable anti-clostridial activity against all tested strains. Results of the agar well diffusion method assays, followed by measurement of MIC and MBC confirmed that the tested strains were more sensitive to ethanolic and methanolic extracts which give the lowest MIC and MBC values (32, 16 respectively) than the watery extracts (512, 256 respectively).

The antimicrobial proprieties of *thymus vulgaris* essential oil could be associated with the thymol content, which has been tested previously and was found to have a significant antibiotic activity (Guarda et al. 2011). Also, the synergistic effect between the different oil compounds, i.e., thymol and carvacrol (Guarda et al. 2011). The mechanism of action of essential oils and their constituents is not fully elucidated. This is complicated by the fact that there are many phyto-chemicals in essential oil and its antibacterial activity may not be attributable to one specific mechanism, but probably there are different targets in the bacterial cell (Burtet al., 2004).

These results were in the same line with other studies where many reports confirmed the high antimicrobial activity (inhibition > 95%) against *Cl. perfringens*; for thyme (*Thymus vulgaris*) alcoholic and watery extract using broth micro dilution methods (Si et al., 2009) and disc diffusion methods (Silva et al., 2014, Kačániová et al., 2014). Also, the antibacterial activity of thyme (*Thymus vulgaris*) extracts was fortified by the results obtained by Radaelliet al. (2016) for MIC and MBC using the microdilution method where the MIC for the essential oil from leaves of *Thymus vulgaris* was 1.25 mg mL^{-1} and showed bactericidal activity at the same value.

Some researchers clarified that the antimicrobial properties of *Thymus vulgaris* extracts against different microorganisms showed inhibitory effect with increasing of concentration, the antimicrobial properties was enhanced (Dobre et al., 2011; Ismail et al., 2012 ; Priti et al., 2012). Our present study is in agreement with other researchers. Therefore, it can be concluded that the Thymus extract inhibitory effect, similar to other antimicrobial compounds, is directly correlated to concentration.

(Sandasi., 2008) reported that the antimicrobial effect of *Thymus vulgaris* alcoholic extract was more than aqueous extract. The present study is in agreement with study that the antimicrobial potential of *Thymus vulgaris* is confirmed and the alcoholic (methanolic and ethanolic) extraction of this plant are suitable choices and more effective than watery extract of the same plant.

The study carried out by Gonçalves et al. (2011) confirmed the considerable antibacterial inhibitory effect of *Thymus vulgaris* extracts was higher when these extracts dissolved in alcohol (ethanol) compared to thyme oil. This may be due to the ability of ethanol to dissolve the polar compounds compared to essential oil. Our obtained results agreed with Gonçalves et al. (2011) who reported that, alcoholic extract is a better option to access thyme extracts with higher antimicrobial efficiency. But disagreed in that methanolic extract of *thymus vulgaris* more effective than ethanolic extract.

5. CONCULOSIONS

Clostridium Perfringens can be isolated from diseased as well as apparently healthy chicken. *Cl. Perfringens* type A is the main cause of necrotic enteritis in chicken as confirmed by PCR results. It is advisable to use different thyme extracts as a natural alternative of chemical antibiotic as it confirmed its antibacterial activity against *Cl. perfringens* specially the methanolic extract where it gave the lowest MIC, MBC and the largest inhibition zone. Consequently, can avoid the risk of antimicrobial resistance associated with the misuse of antibiotics in poultry industry.

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6. REFERENCES

1. Ba - Angood, S.A.; et al. (1996). Azadirachtin ontent of Yemeni neem seed kernels (Azadirachta) india A.(Juss) and its effect on the Development of the Mexican bean beetle Epilachna varivests muls. Univ.of Aden J. of Natural and APL. Sci.1:pp13-25.
2. Balouiri, M., Sadiki, M., and Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis, 6(2), 71–79.
3. Ben-Jabeur ,M., Ghabri, E., Myriam ,M ., and Hamada,W.(2015).Thyme essential oil as a defense inducer of tomato againstgray molf and Fusarium wilt. Plant Physiol. Biochem. 94: 35-40.
4. Burt ,S. (2004). Essential oils: their antibacterial properties and potential applications in foods – a review.
5. Int J Food Microbiol. 94:223–253.
6. Carrasco, J. M., Redondo, L. M., Redondo, E. A., Dominguez, J. E., Chacana, A. P., and Fernandez Miyakawa, M. E. (2016). Use of Plant Extracts as an Effective Manner to Control *Clostridium perfringens* Induced Necrotic Enteritis in Poultry. *BioMed research international*, *2016*, 3278359.
7. CLSI, (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087.
8. Collee, J.G., Fraser, A.G., Marmion, B.Pand Simmons , A.(1996). Practical medical microbiology. 14th education. Churchill Living Stone, New York, 131-151.
9. Diarra , M. S., Block ,G., Rempel , H., Oomah ,B. D., Harrison,J., and McCallum, J., et al. (2013). In vitro and in vivo antibacterial activities of cranberry press cake extracts alone or in combination with beta-lactams against Staphylococcus 10.1186/1472-6882-13-90 .
10. Diarra, M. S.,and Malouin,F. (2014).“Antibiotics in Canadian poultry production and anticipated alternatives,”Frontiers in Microbiology, vol. 5, article 282.
11. Dobre ,A.A., Gagin ,V., and Petru, N.(2011). Antimicrobial activity of essential oils against food-borne bacteria evaluated by two preliminary methods. Rom Biotechol Lett. ; 16:119–125.
12. Emami, N. K., Calik, A., White, M. B., Young, M., and Dalloul, R. A. (2019): Necrotic Enteritis in Broiler Chickens: The Role of Tight Junctions and Mucosal Immune Responses in Alleviating the Effect of the Disease. *Microorganisms, 7*08 231.
13. Engstrom, B.E., Fermer, C., Lindberg, A., Saarinen, E., Baverud, V. and Gunnarsson, A. (2003). Molecular typing of isolates of Clostridium perfringens from healthy and diseased poultry. Vet Microbiol., 94:225–235.
14. Ervelio ,Olazábal., Roberto, González., Sailí ,Flores., and Lizzett ,Alcina.(2005). Efectividad de diferentes combinaciones de tratamientos con antibioticos en la mortalidad por

Clostridium Perfringens en una empresa avicola," *Revista Elecrtonica de Veterinaria*, vol. 6, no. 2, pp. 1–9.

15. Ficken, M.D.,and Wagesm, D.P. (1997). – Necrotic enteritis. In Diseases of poultry, 10th Ed (B.W. Calnek, ed.). Iowa State University Press, Ames, Iowa, 261–264.
16. Ghoneim, N.H. and Hamza, D.A. (2017): Epidemiological studies on Clostridium perfringens food poisonin in retail foods. Rev.Sci.Tech.Off.Int.Epiz., 36 (3): 1-17.
17. Gonçalves,G.M.S., Bottaro, M., Nilson,A.C.(2011). Effect of the Thymus vulgaris essential oil on the growth of Streptococcus mutans. J Basic Appl Pharmaceutical Sci32:375–380.
18. Guarda, A., Rubilar, J.F., Miltz, J., and Galotto, M.J. (2011). The antimicrobial activity of microencapsulated thymoland carvacrol. Int J Food Microbiol. 146:144–150.
19. Harborne,T.B.(1973).Phytochemicalmethods.Halastedpress.Johnwiely and Sons,NewYork.178.
20. Hashemi, S.R.,and Davoodi, H.(2010): Phylogenies as new class of feed additive in poultry industry,"Journal of Animal and Veterinary Advances, vol. 9, no. 17, pp. 2295–2304.
21. Ismail, M.M., Essam, T.M., Mohamed, A.F., and Mourad, F.E. (2012). Screening for the antimicrobial activities of alcoholic and aqueous extracts of some common spices in Egypt. Int J Microbiol Res.; 3:200–207.
22. Jorgensen, J.H., and Ferraro, M.J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin. Infect. Dis., 1749-1755.
23. Kačániová, M.; Vukovič, N.; Horská, E.; Salamon, I.; Bobková, A.; Hleba,L.; Mellen, M.; Vatľák, A.; Petrová , J.;and Bobko, M. (2014) Antibacterial activity against Clostridium genus and antiradicalactivity of the essential oils from different origin, Journal of Environmental Science and Health, Part B: Pesticides, FoodContaminants, and Agricultural Wastes, 49:7, 505-512.
24. Kumar, N. P., Kumar, N. V., and Karthik, A. (2019): Molecular detection and characterization of Clostridium perfringens toxin genes causing necrotic enteritis in broiler chickens. Tropical Animal Health and Production 51:1559–1569.
25. McDevitt ,R.M., Brooker, J.D., Acamovic, T.,and Sparks, N.H.C. (2006). – Necrotic enteritis; a continuing challenge for the poultry industry. World's Poult. Sci. J., 62 (2), 221–247.
26. Nabavi, S.M., Marchese, A., Izadi ,M., Curti ,V., Daglia, M., and Nabavi, S.F.(2015). Plants belonging to the genous Thymus asantibacterial agents: from farm to pharmacy. Food Chem. 173:339-347.
27. Park, J. Y., Kim, S., Oh, J. Y.,Kim, H. R.,Jang, I.,Lee, H. S ., and Kwon,Y. K.(2015). Characterization of *Clostridium perfringens* isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poult. Sci. 94:1158–1164.

Petit, L., Gibert, M., and Popoff, M.R. (1999). Clostridium perfringenstoxinotype and genotype. Trends in Microbiology. 7(3): 104–110.

Priti ,V. (2012). Use of essential oils against gram negative pathogens. J Drug Deliv Therap.; 2:134–137.

Radaelli, M., da Silva, B. P., Weidlich, L., Hoehne, L., Flach, A., da Costa, L. A. M. A., and Ethur, E. M. (2016). Antimicrobial activities of six essential oils commonly used as condiments in Brazil against Clostridium perfringens. Brazilian Journal of Microbiology, 47(2), 424–430.

Rasooli, I., Rezaei, M.B., and Allameh, A. (2006): Ultrastructuralstudies on antimicrobial efficacy of thyme essential oils on Listeria monocytogenes. Int. J. Infect. Dis. 10: 236-241.

Redondo, L. M., Dominguez, J. E., Rabinovitz, B. C., Redondo, E. A., and Fern´andez Miyakawa,M. E. (2015)."Hydrolyzable and condensed tannins resistance in *Clostridium perfringens*,"*Anaerobe*, vol. 34, pp. 139–145.

Sandasi, M., Leonard, C.M., Viljoen, A.M., (2008). The effect of five common essential oil components on Listeria monocytogenes biofilms. Food Control19, 1070–1075

Si, W.; Ni, X.; Gong, J.; Yu, H.; Tsao, R.; Han, Y.; and Chambers, J.R. (2009): Antimicrobial activity of essential oils and structurally related synthetic food additives towards Clostridium perfringens. J. Appl. Microbiol. . 106, 213–220.

Silva, R.O.S., Francisco, C.F.J., Marcus,V.R.M., Carlos, A.O.J., and Nelson, R.M.(2014).Genotyping and antimicrobial susceptibility of Clostridium perfringens isolated from Tinamidae, Cracidae and Ramphastidae species. Brazil Cienc Rural.;44:486–491.

Songer, J. G. (1996). Clostridial enteric diseases of domestic animals, *Clinical Microbiology Review*s, 216–234.

Van Asten, A.J., van der Wiel, C.W., Nikolaou, G., Houwers, D.J., and Grone, A.A. (2009). Multiplex PCR for toxin typing of C. perfringens. Vet Microbiol; 136:411–412.

Vondruskova, H., Slamova, R., Trckova, M., Zraly, Z., and Pavlik,I. (2010).Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review,"Veterinarni Medicin,. 199–224.

Ultee, A., Bennik, M. H. J., and Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. *Applied and Environmental Microbiology*, *68*(4), 1561-1568. https://doi.org/10.1128/AEM.68.4.1561-1568.2002

Yoo, H. S., Lee, S. U.,Park, K. Y.,and Park. Y. H. (1997). Moleculartyping and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. J. Clin. Microbiol. 35:228–232.