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In vitro assessment of *Leuconostoc mesenteroides* zinc nanoparticles against *Salmonella* serovars recovered from broilers chickens

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***Salmonella* serovars are responsible for a variety of acute and chronic diseases in poultry. *Leuconostoc mesenteroides* probiotic can exert antimicrobial activity by producing diverse fermentative metabolites with bactericidal or bacteriostatic activities such as lactic and acetic acids, fatty acids, hydrogen peroxide or diacetyl and antimicrobial proteins such as bacteriocins and peptidoglycan hydrolase enzymes. This trial aimed at adding novel therapy against virulent and multidrug resistance avian salmonellosis. The incidence of salmonellae in this study was 20% with high recovery rate from liver followed by yolk sac and the most common serovars were *Salmonella* Kentukey, *Salmonella* Infantis and *Salmonella* Enteritidis whose antibiogram showed high resistance to ampicillin, nalidexic acid, sulphamethoxasole + trimethoprim and tetracyclines. Selected virulent and multidrug resistant *Salmonella* serovars were exposed to probiotic mixture consisting of *L. mesenteroides* and zinc nanoparticles in different concentration to detect the antibacterial effect against different *Salmonella* serovars as novel therapy for avian salmonellosis. This study revealed that green synthesis of zinc nanoparticles by using *L. mesenteroides* biodegradation 100 nm in size and 10 µg/ml in concentration has potent inhibitory effect against broad range of *Salmonella* serovars but its salmonicidal effect occurred only at 2000 µg/ml.**

Key words: Broilers chicken, *Salmonella*, *Leuconostoc mesenteroides*, zinc nanoparticles.

INTRODUCTION

Avian salmonellosis can develop as a result of infection with poultry-specific serovars, causing systemic illness in birds (Gast, 2003). Great attention has been paid to bacterial resistance to antibiotics for its adverse impacts on morbidity and mortality from diseases caused by resistant bacteria, economic costs of therapy and high risks of the spread of resistant strains among animals and humans (White et al., 2001). Probiotics are defined

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as viable microorganisms, which in sufficient numbers, alter the microbiota of a host body compartment and thereby exert beneficial health effects (Shida-Nanno, 2008). The use of probiotics in enhancing intestinal health has been proposed for many years through several postulated mechanism including competition for limited nutrients, inhibition of the epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, the production of antimicrobial substances and/or the stimulation of mucosal immunity (Servin and Coconnier, 2003). Lactic acid bacteria (LAB) are regarded as a major group of probiotic bacteria.

They are usually described as Gram-positive bacteria, devoid of cytochromes and preferring anaerobic conditions, but are aerotolerant, fastidious, acid-tolerant and strictly fermentative, producing lactic acid as a main product. The most important genera are: *Lactobacillus, Lactococcus, Enterocococcus, Streptococcus, Pediococcus, Leuconostoc* and *Bifidobacterium* (Vasiljevic and Shah, 2008; Perez et al., 2014). *Leuconostoc mesenteroides*, a member of the LAB occurs in several naturally fermented foods and known to produce biodegradable glucose polymer dextran that has wide range of applications in food, cosmetics, pharmaceutical and oil industries (Aman et al., 2012). *L. mesenteroides* is known to produce both water soluble and insoluble dextran (Shukla et al., 2011). While importance of zinc as an essential nutrient has been recognized for many years, only recently, researchers understood the full impact of this nutrient on animal health as they identified 200 zinc dependent enzymes in all the major biochemical pathways in the body (Case and Carlson, 2002).

Nanotechnology has opened the way for introduction of functional nanostructures which can be used as building blocks to create novel finding such as antimicrobial biodegradable materials that is effective against a variety of pathogens including Gram negative pathogens, so the current study aimed to investigate the inhibitory effect of *L. mesenteroides* Zn-NPs on broiler chicken *Salmonella* serovars.

MATERIALS AND METHODS

Isolation, identification, virulence and antibiotic resistance profile of *Salmonella* serovars from broiler chickens

Under complete sterile condition, broilers internal organs including liver, yolk sac, lung, caecum and spleen were examined for isolation and identification of Salmonellae according to (ISO, 2002); the recovered isolates were serotyped in the Central Laboratory for Quality Control on Poultry Production (CLQP) in Dokki, Giza, Egypt, according to Kauffmann and Das Kauffmann (2001). The antibiogram disk diffusion technique was adapted according to CLSI (2017).

Green synthesis of Zn-NPs using *L. mesenteroides* probiotic

L. mesenteroides NRRL B-1149 was propagated as stab in MRS agar medium at 30°C according to Goyal and Katiyar (1996) as used in biodegradation of zinc sulphate as a substrate for production of Zn-NPs sized 100 nm according to Otari et al. (2012) with slight modification and characterized in the central laboratory of elemental and isotopic analysis, nuclear research center, atomic energy authority, Egypt according to the technique of Mashrai et al. (2017).

In vitro assessment of *L. mesentroids* Zn-NPs against *Salmonella* serovars

Selected virulent and multidrug resistant *Salmonella* serovars were cultured in Tryptic soya broth and incubated at 30°C for 24 h. In order to examine the antibacterial activity of the Zn-NP on *Salmonella* serovars, ZnO nanoparticles were suspended in sterile normal saline and constantly stirring until a uniform colloidal suspension. Agar diffusion method was carried out according to Perez et al. (1990) and determination of minimum inhibitory concentration and minimum bactericidal concentration (MIC/MBC) as antimicrobial activity nano-ZnO according to Chwalibog et al. (2010).

RESULTS

Incidence and antibiogram of broilers chickens Salmonella

From the result of this study, the incidence of *Salmonella* in broilers chickens as shown in Table 1 were 21% with high recovery rate from liver followed by yolk sac. Serotyping of recovered *Salmonella* isolates in Table 2 revealed that the most common serovars were *Salmonella* Kentukey, *Salmonella* Infantis and *Salmonella* Enteritidis whose antibiogram as shown in Table 3 high resistance to ampicillin (90%), nalidexic acid (88%), sulphamethoxasole + trimethoprim (82%) and tetracyclines (82%).

Effect of *L. mesenteroides* zinc nanoparticles on broilers chickens *Salmonella*

The selected virulent and multidrug resistant *Salmonella* serovars in the present study were exposed to *L. mesenteroides* zinc nanoparticles (Figure 1) in different concentrations to detect its antibacterial effect as novel therapy for avian salmonellosis. The results (Table 4) revealed that zinc nanoparticles 100 nm in size and 10 µg/ml in concentration has potent inhibitory effect against broad range of *Salmonella* serovars but its salmonicidal effect occurred only at 2000 µg/ml as shown in Figure 2) with destruction of *Salmonella* cell wall after treatment with nanoparticles.

DISCUSSION

Salmonellosis in poultry is a worldwide problem both for poultry and as a vehicle for human disease (Sharp,

Table 1. Incidence of *Salmonella* serovars in broiler chickens organs.

Organ	N = samples	n= positive	%	All %
Liver	45	14	31(14/45)	5.8(14/240)
Yolk Sac	60	12	20(12/60)	5(12/240)
Lung	40	4	10(4/40)	1.6(4/240)
Caecum	50	12	24(12/50)	5(12/240)
Spleen	45	8	17.7(8/45)	3.3(8/240)
Total	240	50	--	20.7%(50/240)

Table 2. Recovery of *Salmonella* serovars isolated from broilers chickens organs.

Organs	Serotypes	Number of serovars	%
	S. Kentucky	7/14	50
	S. Enteritidis	4/14	29
Liver	*S.* Pullorum	1/14	7
	S. Infantis	1/14	7
	S. Newport	1/14	7
	S. Kentucky	5/12	42
	*S.*Enteritidis	2/12	17
Yolk sac	*S.* Heidelberg	2/12	17
	S. Infantis	2/12	17
	S. Virginia	1/12	8
	S. Hiedelberg	2/4	50
Lung	*S.* Labadi	1/4	25
	S. Infantis	1/4	25
	S. Kentucky	7/12	58
Caecum	*S.* Typhi	1/12	8
	S. Infantis	4/12	33
	S. Kentucky	6/8	75
Spleen	*S.* Agona	1/8	12.5
	S. Infantis	1/8	12.5

1991). Pathogenesis of *Salmonella* depends on its ability to survive and replicate inside host cells. This virulence trait is linked to the ability to cause systemic infections and a large number of genes are required to enable *Salmonella* to cope with nutritional limitations, to avoid clearance by the host immune system or survive damage by antimicrobial peptides and radicals (Hegazy and Hensel, 2012). The occurrence of *Salmonella* Enteritidis has significant increase in poultry carcasses from 2000 to 2005 in the US. Studies between 2000 and 2009 showed that the predominance of *Salmonella* serovar in poultry was *Salmonella* Enteritidis, which was resistant to multiple antibiotics, including marked resistance to third generation cephalosporins. In the past years in the US, increased resistance to Ceftiofur was observed in poultry strains. In 1997, resistance to this antibiotic was 1.6%, and in 2003, it was 7.4% (Medeiros, 2011; Voss-Rech et al., 2015). In the current investigation, incidence of *Salmonella* in broiler chickens shown in Table 1 were 21% with high recovery rate from liver followed by yolk sac. Serotyping of recovered *Salmonella* isolates in Table 2 revealed that the most common serovars were *S.* Kentukey, *S.* Infantis and *S.* Enteritidis with antibiogram shown in Table 3 with high resistance to ampicillin (90%), nalidexic acid (88%), sulphamethoxasole + trimethoprim (82%) and tetracyclines (82%). Antibiotic resistance mechanisms can be categorized as (i) modification or destruction of the antimicrobial agent, (ii) pumping the

Table 3. Resistance pattern of *Salmonella* serovars recovered from broiler chickens.

Antimicrobial agents	Resistance patterns					
	R*	%	I*	%	S*	%
Sulphamethaxole + Trimethoprim (SXT)	41/50	82	0/50	0	9/50	18
Amikacin 30 µg	0/50	0	2/50	4	48/50	96
Imepenem 10 µg	0/50	0	3/50	6	47/50	94
Tetracyclines 30 µg	41/50	82	1/50	2	8/50	16
Ampicillin 10 µg	45/50	90	1/50	2	4/50	8
Nalidixic acid 30 µg	44/50	88	1/50	2	5/50	10
Chloramphenicol 30 µg	21/50	42	2/50	4	27/50	54
Gentamicin 10 µg	1/50	2	3/50	6	46/50	92
Ciprofloxacin 5 µg	29/50	58	17/50	34	4/50	8
Azetreonam 30 µg	8/50	16	3/50	6	39/50	78
Ampicillin + Sulbactam 20 µg	12/50	24	4/50	8	34/50	68
Cefepem 30 µg	8/50	16	2/50	4	40/50	80
Ceftriaxone 30 µg	7/50	14	5/50	10	38/50	76
Cephalothin 30 µg	16/50	32	12/50	24	22/50	44
Cefotaxime 30 µg	4/50	8	0/50	0	46/50	92
Ceftazidem 30 µg	4/50	8	0/50	0	46/50	92

*R: Resistant, I: intermediate, S: sensitive.

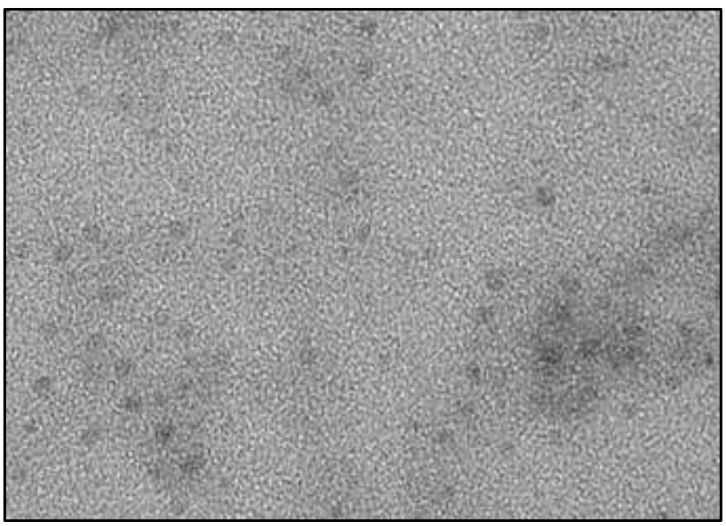

Figure 1. SEM images of *Leuconostic* Zn-Nps.

antimicrobial agent out from the cell by efflux pumps, (iii) modification or replacement of the antibiotic target and (iv) decrease in cell membrane permeability. Walsh (2003) also showed that resistance to antibiotics is due to temporary or permanent change of bacterial genetic information. Most resistance genes are found in plasmids. Acquired resistance is caused by the transfer of resistance genes from one cell to another (Tavares, 2001).

Currently nanotechnology has the potential to impact many aspects as: food security, disease treatment delivery system, new tools in cellular and molecular biology, new materials for pathogens detection (Weiss et al., 2006). Recent studies showed that nanoparticles particularly, zinc oxide had selective toxicity to micro-organisms (Reddy, 2007). The study is on evaluation of prepared *L. mesentroides* zinc nanoparticles as potent agent against broilers chickens Salmonellae; thus, selected *Salmonella* serovars which is virulent and multidrug resistant were exposed to different concentration of this molecules to detect its antibacterial effect against avian salmonellosis; the result in Table 4

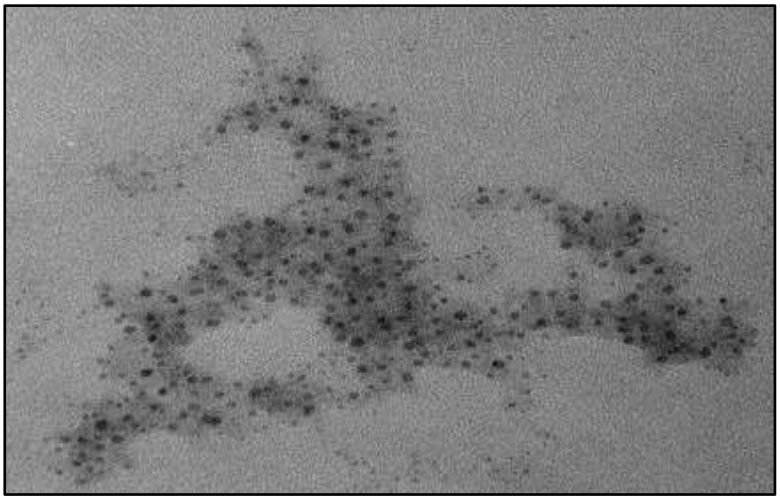

Figure 2. SEM images of *Salmonella* after exposure to *Leuconostic* Zn-Nps (complete damage of *Salmonella*).

Table 4. Anti-*Salmonella* effect of *L. mesentroids* Zn-NPs.

Zn-Nps concentration (μg/mL)	Agar diffusion methods (zone of inhibition of *Salmonella*) (mm)	Minimum inhibitory concentration, MIC (μg/ml)	Minimum bactericidal concentration, MBC (μg/ml)
10	8.5±1.24	< 10	1000
20	12.3±1.54	< 10	1000
40	14.7±1.34	< 10	1000
60	15.5±1.65	< 10	2000
80	16.2±1.22	< 5	2000
100	17.5±1.55	< 5	2000

showed that zinc nanoparticles 100 nm in size and 10 μg/ml in concentration has potent inhibitory effect against broad range of *Salmonella* serovars but its salmonicidal effect occurred only at 2000 μg/ml. Advances in the field of nanosciences and nanotechnology have brought to form nanosized inorganic and organic particles in medicine and therapeutics (Gajjar et al., 2009). Antimicrobial effect of zinc nanoparticles (Zn-NPs) occurs by different ways such as: formation of H_2O_2 which retard microbial growth, another way is by releasing of Zn^{+2} which damage microbial cell membrane and interact with intracellular contents (Moraru et al., 2003), while Violeta et al. (2011) attributed the antimicrobial activities of Zn-NPs to photocatalytic production of reactive oxygen species that damage cell components and interrupt energy transduction. Recently, new safe antimicrobial agents were needed to prevent and overcome bacterial infections. The large increase in the number and occurrence of antibiotic resistant bacterial strains has prompted a renewed interest in the use of metals as antibacterial agent (Odds et al., 2003).

Conclusion

This study suggests that broilers play a potential role as a reservoir of multi drug resistant and virulent *Salmonella* serovars with special reference to novel control methods by lactic acid bacteria (LAB) as *L. mesenteroides* zinc nanoparticles; the molecule proved as *in vitro* inhibitory agent for *Salmonella* in broiler chickens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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