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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Inhibition of *Clostridium perfringens* LMG 11264 in Meat Samples of Chicken Turkey and Beef by the Bacteriocin Plantaricin UG1

Gamal Enan
Department of Sciences, Laboratory of Microbiology, King Khalid Military Academy,
Box: 22140, Riyadh 11495, Saudi Arabia

Abstract: The inhibition of *Clostridium perfringens* LMG 11264 (*Cl. perfringens*) by the bacteriocin plantaricin UG1 *in vitro* (brain heart infusion broth and cooked meat broth) and in meat samples (cooked meat, uncooked meat & dry mortadella) of poultry (chicken and turkey) and beef was studied. The inhibitory activity of plantaricin UG1 was apparently more in liquid systems (BHI broth & cooked meat broth) than in meat samples. The viable cells of *Cl. perfringens* were increased from 10^5 cfu/gm to 10^7 - 10^9 cfu/gm in almost all controls, but where decreased from 10^5 cfu/gm to zero in the bacteriocin treated samples of cooked and uncooked chicken, turkey and beef within one week. Addition of partially purified plantaricin UG1 to mortadella samples of chicken, turkey and beef resulted in decline of viable cell counts of *Cl. perfringens* form 10^5 cfu/gm to zero within two weeks. The inactivation power of plantaricin UG1 against *Cl. perfringens* endospores was shown in distilled water.

Key words: *Clostridium perfringens* (*Cl. perfringens*), bacteriocin, plantaricin UG1, inhibition, meat samples

Introduction

Clostridium perfringens is a gram - positive, non-motile spore forming anaerobic rod. The *Cl. perfringens* strains are widely distributed in soil, sewage, stool samples and intestines of animal and humans (Steele and Wright, 2001). Anyone can get *Cl. perfringens* food poisoning by eating from common food stored in large quantities at room temperature at schools, camps, bankets and buffets (Martel *et al.,* 2004). The symptoms which include watery diarrhea, intense abdominal pain, gas gangrene and inflammation, have been attributed to a protein entertoxin produced by the organism in the intestine (Teo and Tan, 2005). The neurotoxin causes also necrotic enteritis in animals and humans (Wise and Siragusa, 2005). To prevent *Cl. perfringens* infection, leftover cooked beef and poultry meat should be refrigerated promptly and reheated thoroughly (internal temperature, 75°C) before serving. Traditional methods used for protection of the fermented beef and poultry products against bacterial pathogens are addition of chemical preservatives which are human harmful. Hence, inhibition of clostridia in foods by a safe lactic acid bacterium or its metabolites is of interest. In this regard the bacteriocin plantaricin UG1 produced by *Lactobacillus plantarum* UG1 isolated from dry sausage inhibited some food-borne pathogens including *Cl. perfringens in virto* (Enan *et al.,* 1996; Enan, 2000; Enan *et al.*, 2002; Enan, 2006). Plantaricin UG1 was active against clostridia at acidic and neutral pH values (pH 4-7) and over a wide temperature range (zero-90°C) (Enan *et al.*, 1994a,b; Enan *et al.*, 2004).
Nothing is known in the literature about inhibition of clostridia by bacteriocin of *Lactobacillus plantarum.* In an attempt to fill this gap, the present work was undertaken to study the inhibition of *Cl. perfringens* viable cells by plantaricin UG1 in culture media and in samples of uncooked meat, cooked meat and dry mortadella of chicken, turkey and beef. In addition the survival of *Cl. perfringens* endospores in distilled water treated with plantaricin UG1 was investigated.

Materials and Methods

Bacterial strains and culture media: *Cl. perfringens* LMG11264 was kindly provided from LMG culture collection, Laboratorium voor Mikrobiologie, Universiteit Gent, Belgium. Its spores were maintained as frozen stocks in glass beads at -20°C. Few glass beads were suspended in cooked meat broth (Difco), heated at 80°C for 15 min to stimulate spore germination (Smith, 1975) and were incubated at 37°C for 48 h. Cells were then subcultured every 48 h in cooked meat broth. *Lactobacillus plantarum* UG1, the producer of plantaricin UG1 was provided from Department of Food Technology and Food Preservation, Faculty of Agricultural and Applied Biological Sciences, University of Gent, Belgium. It was maintained at -20°C in De Man Rogosa and Sharpe medium (De Man *et al.*, 1960) plus 20 % glycerol (Joerger and Klaenhammer, 1986) and was propagated in MRS broth (Abdel-Salam *et al.*, 2003).

Preparation of partially purified plantaricin UG1: *L. plantarum* UG1, the producer of plantaricin UG1, was grown in MRS broth for 16 h at 30°C. Cell free supernatant was obtained by centrifuging the culture

Permanent address: Department of Botany, Faculty of Science, Zagazig University, Zagazig, Egypt

(10000xg for 10 min at 4°C). pH value of cell free supernatant was then adjusted at pH 6.5. This is to exclude the inhibitory activity due to organic acids (Enan *et al*., 1996; Enan and Saad, 2005). This pH-adjusted cell free supernatant was subjected to ammonium sulphate precipitation as described previously (Bhunia *et al*., 1988). The ammonium sulphate precipitates (surface pellicels and pellets) were recovered in 10m M potassium phosphate buffer pH 6.5 and dialyzed against the same buffer for 24 h at 4°C in visking dialysis membrane (Enan *et al*., 2004). This partially purified plantaricin UG1 was sterilized by filtration through cellulose membrane filters (Amicon, 0.45µm) and was used in the experiments. 1ml of this partially purified plantaricin UG1 appeared to contain 2020 AU/ml as assayed previously (Enan *et al*., 1996) with *Cl. perfringens* as the indicator organism.

Food samples: Meat slices of fresh raw chicken, turkey and beef were prepared. They were minced by their grinding in sterile mincer. Half of minced amounts were cooked in steam oven. 100 g portions of cooked samples and 100 g portions of uncooked samples were placed aseptically in sterile plastic bags and were used in the experiments. Brain heart infusion broth (BHI, Difco) and cooked meat broth samples (Difco) were added into 250 ml screw capped bottles (100 ml for each), autoclaved and were used in the experiments. Dry samples of chicken mortadella, turkey mortadella and beef mortadella (National Co. Foods, Saudi Arabia) were cut by sterile knife; then 100 g portions were placed aseptically in sterile plastic bags and were used in the experiments.

Inhibition of *Cl. prefringens* LMG 11264 *in vitro* and in meat samples of plantaricin UG1: The meat samples tested (100 g of each) and 100 ml aliquots of either brain heart infusion broth or cooked meat broth were inoculated by cell suspension of the experimental *Cl. perfringens* strain to give 10^5 cfu/gm or ml final concentration, and then shaken vigorously by hand to distribute the inocula. The above inoculated samples were treated with 40880 AU/ml partially purified plantaricin UG1 and were shaken again to mix the bacteriocin with the inoculated samples. The air was excluded from bags by hand; and from culture media as their boiling during sterilization drive off dissolved oxygen (Rhodehamel and Harmon, 2006). Control samples were inoculated by *Cl. perfringens* cells, but not treated with the bacteriocin plantaricin UG1. Samples and controls were incubated at 37°C in an anaerobic jars equipped with GasPak H_2 + CO_2 generator envelops and catalyst as described by the manufacturer's instructions (Oxiod). After appropriate time intervals and controls were taken and analyzed for viable counts of *Cl. perfringens* as described previously (Garcia *et al*., 2001; Rhodehamel and Harmon, 2006).

Preparation of *Cl. perfringens* spores: This was performed by the procedure described by Craven and Blankenship (1985). About 1% inoculum of *Cl. perfringens* was inoculated into Ducan-Strong Sporulation medium (Duncan and Strong, 1968). Incubation was carried out at 37°C for 24 h without provisions for anaerobiosis. Spores were harvested by centrifugation at 4°C and cleaned by repeated washing with cold deionizer water. The cleaned phase dark spores were used in the experiments.

Survival of *Cl. perfringens* spores in the presence of plantaricin UG1: A series of test tubes, each containing 10 ml distal water were inoculated with 10^7 spore/ml final concentration and was treated with 10200 AU/ml. Controls were spores suspensions without bacteriocin. Samples and controls were incubated under anaerobic conditions as described previously by Rhodehamel and Harmon (2006) for 7 days. Every day, two tubes (one tube of samples and one tube of controls) were taken and heat shocked by their heating at 80°C for 15 min. The colony forming units of the heat shocked samples and controls were determined as described previously (Kang *et al*., 1969; Garcia *et al*., 2001).
Such values of cfu /ml are equivalent to the values of survived spore/ml (Johnson *et al*., 1982). The percentage of survived spores = (survived spore/ml in the treated sample ÷ (survived spore/ml in control) x 100. The percentage of inactivated spores = 100% - percentage of survival (Enan and Saad, 2005).

Results

The effect of the bacteriocin plantaricin UG1 on growth of the *Cl. perfringens* viable cells in both cooked meat broth and brain heart infusion broth is shown in Table 1. The viable cell population of *Cl. perfringens* were increased from 10^5 cfu/ml to 6.6 x 10^8 cfu/ml within 60 h in controls but were decreased from 10^5 cfu/ml; 10^5 cfu/ml to 3.2 x 10^3 cfu/ml; 1.2 x 10^3 cfu/ml in cooked meat broth; brain heart infusion broth treated with the bacteriocin plantaricin UG1 respectively within 24 h. No viable

Table 1: Inhibition of the *Cl. perfringens* LGM 11264 viable cells by plantaricin UG1 in culture media

Time (h)	Colony forming units (cfu/ml) of the *Cl. perfringens* LMG 11264 viable cells in the treated samples and Controls (media without bacteriocin)			
	Cooked meat broth		Brain heart infusion broth	
	Control	Sample	Control	Sample
Zero	10^5	10^5	10^5	10^5
6	$9.1x10^5$	10^5	$6.6x10^5$	10^5
12	$3.2x10^6$	$2.1x10^4$	$1x10^6$	$3.3x10^4$
18	$8.7x10^7$	$1x10^4$	$8.6x10^6$	$8x10^3$
24	$7x10^8$	$3.2x10^3$	$2.2x10^7$	$1.2x10^3$
36	$8.6x10^8$	$1.1x10^3$	$8.1x10^8$	$2.7x10^2$
48	$1.1x10^9$	$2x10^2$	$9.7x10^8$	$1x10^2$
60	$6.6x10^8$	zero	$1x10^9$	zero

Table 2: Growth of the *Cl. perfringens* LMG 11264 viable cells in uncooked meat (chicken, turkey and beef) and its control by the bacteriocin plantaricin UG1

Time (h)	Colony forming units (cfu/gm) of *Cl. perfringens* LMG11264 in the treated uncooked meat samples (chicken, turkey, and beef) and controls (uncooked meat without bacteriocin)					
	Uncooked meat of chicken		Uncooked meat of turkey		Uncooked meat of beef	
	Control	Sample	Control	Sample	Control	Sample
Zero	10^5	10^5	10^5	10^5	10^5	10^5
6	$4.1x10^5$	10^5	$6.2x10^5$	10^5	$3.3x10^5$	10^5
12	$7.1x10^5$	$7.8x10^4$	$9.2x10^5$	$8.6x10^4$	$6.2x10^5$	10^5
18	$9.1x10^5$	$5.2x10^4$	$3.1x10^6$	$6.7x10^4$	$8.1x10^5$	$8.1x10^4$
24	$2.2x10^6$	$3.1x10^4$	$8.1x10^6$	$4.4x10^4$	$1.1x10^6$	$7.2x10^4$
36	$6.6x10^6$	$1.1x10^4$	$2.2x10^7$	$2.7x10^4$	$6.2x10^6$	$5 x 10^4$
48	$2.2x10^7$	$7x10^3$	$6.2x10^7$	$9.2x10^3$	$9.2x10^6$	$3.2x10^4$
60	$5.6x10^7$	$5x10^3$	$8x10^7$	$8x10^3$	$2.1x10^7$	$1.1x10^4$

counts of *Cl. perfringens* were observed in the bacteriocin treated culture media after 60 h of incubation. Table 2 shows the growth values (cfu/gm) of *Cl. perfringens* in uncooked meat samples of chicken, turkey and beef. *Cl. perfringens* grew in controls and its viable cells increased from 10^5 cfu/ml to 2-8 x 10^7 cfu/ml within 60 h of incubation. However, in the bacteriocin treated samples, the viable cell population of *Cl. perfringens* decreased gradually reaching 5 x 10^3 cfu/gm; 8 x 10^3 cfu/gm; 1.1 x 10^4 cfu/gm after 60 h in chicken; turkey; beef respectively. No viable cells were being observed after 7 days of storage.

Addition of partially purified plantaricin UG1 to cooked meat samples resulted in a gradual decreased of the *Cl. perfringens* viable cells reaching 10^4 cfu/gm; $1.7x10^3$ cfu/gm; 9.2 x 10^3 cfu/gm after 24 h and 2 $x10^2$ cfu/ml; zero; $1x10^3$ after 60 h in chicken meat; turkey meat; beef meat respectively (Table 3). However, in controls, viable cell counts of *Cl. perfringens* were increased from 10^5cfu/gm to almost 10^9 cfu/ml (Table 3). No viable cells were obtained in the bacteriocin treated samples after 7 days of storage.

The effect of partially purified plantaricin UG1 on the growth of *Cl. perfringens* in mortadella samples (chicken, turkey and beef) is shown in Table 4. The growth values in controls were increased from 10^5 cfu/gm to almost 1-3 x 10^7 cfu/gm within 60 h. However, in the bacteriocin treated samples, viable cell populations of *Cl. perfringens* were decreased gradually reaching 7.2 x 10^3 cfu / gm; 1.2 x 10^7 cfu / gm; $2x10^3$ cfu/gm in chicken mortadella; turkey mortadella; beef mortadella respectively (Table 4). No growth of the experimental clostridia was obtained in the bacteriocin treated mortadella samples after further two weeks of storage.

To start work about the effect of bacteriocin plantaricin UG1 on the *Cl. perfringens* spores, the experimental clostridia spores were prepared as described in Materials and Methods and resuspended in distilled water treated with the bacteriocin plantaricin UG1. Results are shown in Table 5. In control samples, about 3% decrease (from 10^5 spore/ml to 9.4 x 10^4 spore/ml) was observed in number of spore/ml within 7 days. However, in distilled water treated with partially purified plantaricin UG1, values of residual spores were shown to be 9.8 x 10^3 spore/ml (almost 90% decrease in spore/ml) after 7 days of storage. This showed that the percentage values of survived spores; inactivated spores were recorded 10.10%; 89.9 respectively (Table 5).

Discussion

Samples of uncooked meat, cooked meat and dry mortadella of chicken, turkey and beef were chosen in this study because those food products were shown to be the main vehicle of *Cl. perfringens* infection in Saudi Arabia (Saad *et al.*, 2000). The *Cl. perfringens* spores can survive cooking temperatures and germinate during post-cook handling (Craven and Blankenship, 1985). Packages of uncooked meat or poultry were shown also to contain the *Cl. perfringens* pathogen (Doyle, 1989). In addition the salt-cured corned beef and mortadella samples of chicken, turkey and beef were also accounted as a source of *Cl. perfringens* illness (Green, 2002).

The experiments employed herein were designed to concur with the conditions happened sometimes for food products in Saudi Arabia when food has been cooked in large quantities and then held for too long at room temperatures (15°C to 45°C) or on a steam table at schools, camps, banquets and buffets. Hence, the meat samples (chicken, turkey, beef) inoculated with *Cl. perfringens* were incubated herein at 37°C which is the optimum growth temperature for *Cl. perfringens* (Johnsson *et al.*, 2004). The bacteriocin plantaricin UG1 concentrations used were chosen because they showed, preliminary, to saturate the adsorption site(s) of the inocula used in this study (Enan, 2006). Spore suspensions were, preliminary, heated at 80°C for 15 min. to stimulate spore germination and this was also used in previous work in this respect (Stewart *et al.*, 1981).

Compared with the inhibition of *Cl.perfringens* in both culture media and meat samples (chicken, turkey and

Table 4: Growth of the *Cl. perfringens* LMG 11264 viable cells in dry sausage samples (chicken mortadella, turkey mortadella, beef mortadella) and its control by the bacteriocin plantaricin UG1

Time (h)	Colony forming units (cfu/gm) of *Cl. perfringens* LMG 11264 in the treated samples and controls					
	Chicken mortadella		Turkey mortadella		Beef mortadella	
	Control	Sample	Control	Sample	Control	Sample
Zero	10^5	10^5	10^5	10^5	10^5	10^5
6	$2.7x10^5$	10^5	$6.1x10^5$	10^5	$4.1x10^5$	10^5
12	$5.7x10^5$	$9.7x10^4$	$8.8x10^5$	$6.2x10^4$	$7x10^5$	$7.2x10^4$
18	$9 x10^6$	$7.7x10^4$	$2.7x10^6$	$3.3x10^4$	$1x10^6$	$5x10^4$
24	$3x10^6$	$5.1x10^4$	$5.6x10^6$	$1.1x10^4$	$6.1x10^6$	$3.1x10^4$
36	$7.1x10^6$	$3.1x10^4$	$8.8x10^6$	$6.2x10^3$	$9.7x10^6$	$8.2x10^3$
48	$9.1x10^6$	$1.1x10^4$	$1x10^7$	$1.3x10^3$	$2.1x10^7$	$6 x10^3$
60	$1.1x10^7$	$7.2x10^3$	$1.2x10^7$	$2x10^2$	$3.2x10^7$	$2 x10^3$

Table 5: Survival of the *Cl. perfringens* LMG 11264 spores in distilled water either treated (samples) or non-treated (controls) with the bacteriocin plantaricin UG1

Time (day)	Values of residual spores/ml in		Percentage Survived spores	Percentage of spores
	Control	Sample		
1	10^5	10^5	100%	zero %
2	10^5	$8.8x10^4$	88%	12 %
3	10^5	$7.4x10^4$	74%	26%
4	$9.8x10^4$	$6.4x10^4$	65.31%	34.69%
5	$9.7x10^4$	$4.3x10^4$	44.32%	55.68%
6	$9.8x10^4$	$3x10^4$	30.6%	69.4%
7	$9.7x10^4$	$9.8x10^3$	10.10%	89.9%

beef), the bacteriocin plantaricin UG1 was less effective in the meat system, because several factors in meat may interfere with the bacteriocin activity such as (i) adsorption of bacteriocin to meat particles and (ii) limited diffusion of bacteriocin in solid meat samples (Enan, 2000; Enan *et al.*, 2002).

Addition of the bacteriocin plantaricin UG1 to samples of uncooked and cooked meat (chicken, turkey, beef) decreased viable cells of *Cl. perfringens* by 100 % within one week. This anticlostridial activity of plantaricin UG1 at 37°C will be quite promising for development of wider applications of this bacteriocin as natural biopreservative for several kinds of meat. Such results are in conform with many authors who recommended the use of the bacteriocin nisin as meat biopreservative (Nettles and Barefoot, 1993). Compared with the applications of both nisin and plantaricin UG1 as natural biopreservatives, plantaricin UG1 could be more effective due to its activity at acidic and neutral pH (Enan *et al.*, 1994 a,b); but nisin is active at only acidic pH values (Varadaraj *et al.*, 1993). Increasing consumer demand for ' natural ' and "additive- free" products has led to greater interest in the application of natural inhibitory substances like bacteriocins as food biopreservatives which could replace or reduce the use of chemical additives (Vaughan *et al.*, 1994).

Interesting growth of *Cl. perfringens* was observed herein at 37°C in mortadella samples of chicken, turkey or beef containing 85% meat (chicken, turkey, or beef), 5% NaCl, 10% spices and other constituents. This is possible since *Cl. perfringens* was reported to survive NaCl concentrations up to = 6 % which were higher than those used in the normal curing of meat (Scott, 1957; Gough and Alford, 1965; Kang *et al.*, 1969). Hence, mortadella samples must be stored at temperatures below 15 °C. where *Cl. perfringens* will not grow (Kramer and Schallehn, 1974). As plantaricin UG1 inhibited *Cl. perfringens* in dry mortadella, it could be used as meat additive and its producer, *Lactobacillus plantarum* UG1, could be used as starter culture for meat fermentation with protection of the meat product against *CL.perfringens* (Enan *et al.*, 1996).

The bacteriocin plantaricin UG1 inactivated 90% of *CL.perfringens* spores within 7 days. This could be due to the bacteriocin effect which can alter the tertiary structure of bacterial endospore making inactivated spores (Keynan *et al.*, 1964, Lopez *et al.*, 2003). The inactivation of *Cl. perfringens* spores noticed *in vitro* in this study is of interest due to the wider applications of the plantaricin UG1 producer, *Lactobacillus plantarum* UG1, as starter culture for meat fermentation. However, further studies will be needed to check the possible inactivation of *Cl. perfringens* spores in foods by the bacteriocin plantaricin UG1.

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