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Control of the Regrowing Bacteriocin Resistant Variants of *Listeria monocytogenes* LMG 10470 *in vitro* and in Food by Nisin-plantaricin UG1 Mixture

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Abstract: Nisin or plantaricin UG1 induced a listericidal action *in vitro* and in food followed by regrowth of the target cells. The regrowing cells of *Listeria monocytogenes* LMG10470 were proved to be resistant variants at a resistance frequency from 10^{-5} to 10^{-6}. The mix of nisin and plantaricin UG1 avoided the regrowth of becteriocin resistant cells of *L. monocytogenes* LMG10470 *in vitro* and in food. No cross resistance was found between nisin and plantaricin UG1 as *Listeria*l cells resistant to one bacteriocin were sensitive to the other one; indicating on a synergistic listericidal effect of nisin-plantaricin UG1 mixture which could extend the shelf life of dairy products, meat products and tomato paste.

Key words: Nisin, plantaricin UG1, bacteriocin, food, resistance, *Listeria monocytogenes*, regrowing variants, bacteriocin mixture

INTRODUCTION

Listeria monocytogenes is a gram positive rod that is catalase positive and shows a characteristic tumbling motility. *Listeria monocytogenes* (*L. monocytogenes*) has long been recognized as a veterinary pathogen and in humans causes a disease known as listeriosis in pregnant women and neonates (Pucci *et al*., 1988). It is found in soil, decaying animals and dead vegetation and in intestinal tracts of over domestic and wild species of birds and animals including sheep, cattle, chickens and swine (Moura *et al*., 1993). The ability of *listeria*s cells to grow at temperatures ranging from 1 to 45°C, their high salt tolerance and their ability to initiate growth at a relatively low pH make these pathogens particularly difficult to control in food (Vignolo *et al*., 2000). A novel approach to the control of *L. monocytogenes* in food is the use of antimicrobial proteins (bacteriocins) from lactic acid bacteria (Nettles and Barefoot, 1993). However, listerias cells can develop tolerance towards nisin and pediocin like bacteriocins *in vitro* and in foods (Rekhif *et al*., 1994). An improved control of the target organisms and inhibition of bacteriocin resistant strains and species can be obtained by using a combination of one or more bacteriocins (Vignolo *et al*., 2000).

The bacteriocin plantaricin UG1 produced by *Lactobacillus plantarum* UG1 (Enan *et al*., 1994a, b) inhibited *L. monocytogenes in vitro* and in food (Enan *et al*., 1996; Enan, 2002). However, few regrowth was observed thereafter (Enan, 2002). The regrowing listerias cells were characterized to be resistant variants at a resistance frequency around 10^{-6} to control the regrowth of bacteriocin resistant cells of *L. monocytogenes* in food system, combined effect of bacteriocins is important (Bouttefroy and Milliere, 2000). The present research was undertaken to study the combined effect of nisin and plantericin UG1 on the growth of the plantaricin UG1-resistant variants of *L. monocytogenes* LMG10470 *in vitro* and in some Saudi foods viz., minced meat, pastenrized milk, kareesh cheese and tomatoes paste.

MATERIALS AND METHODS

Microorganisms and media: *L. monocytogenes* LMG10470 was kindly provided from Department of Food Technology and Food Preservation, Faculty of Agricultural and Applied Biological Sciences, University of Gent, Belgium. It was used as the indicator organism. It was maintained as frozen stocks in trypticase soy broth (Difco) plus 10% glycerol and was subcultured every 2 weeks in trypticase soy broth. Growth values of *L. monocytogenes* were measured on *Listeria* selective agar (Oxoid) as cfu/ml. growth values were taken from three replicates. *Lactobacillus plantarum* UG1 was used as a bacteriocin producer (plantericin UG1 producer) (Enan *et al*., 1994a, b). It was maintained at-20°C in De Man, Rogosa and Sharpe medium (MRS) (De Man *et al*., 1960) and was propagated in MRS broth (Enan *et al*., 1996; Enan, 2000).

Food samples: Samples of minced meat were prepared by their grinding in a sterile mincer. About 100 g portions

were placed aseptically in sterile plastic bags and were used in the experiments. Tomato paste (Sadafco Co., Saudi Arabia) were prepared. About 100 g samples of tomato paste were placed aseptically in 250 mL sterile screw capped bottles and were used in the experiments. Pasteurized milk (Nadec Co., Saudi Arabia) was added aseptically in sterile 250 mL Erlenmeyer flasks (100 mL for each) and was used in the experiments. Kareesh cheese is an Egyptian famous cheese. It was provided from Wella Marketing Company (Saudi Arabia) and was cutted aseptically by sterile knife. Hundred gram portions of Kareesh cheese were placed aseptically in sterile plastic bags and were used in the experiments. This investigation was carried out in Laboratory of Microbiology, Department of Sciences, King Khalid Military Academy, Saudi National Gaured, Saudi Arabia.

Preparation of bacteriocins and bacteriocin bloassay: The bacteriocin nisin was provided from Somatco Chemical Company (Saudi Arabia). Stock nisin solution was prepared by solubilizing appropriate amounts of powder in 0.02 HCl solution. The pH was adjusted to 3.5 with 1N NaOH. The solution was then filter-sterilized (0.22 μm, Milipore). The nisin bioassay was determined by the critical dilution assay (Biswas *et al*., 1991). The *Listeria monocytogenes* LMG10470 was used as the indicator organism. One IU (International Unit) of nisin was showed to be equivalent to 0.20 AU (Arbitarary Unit). So, 1 mL of nisin solution containing 10^4 IU was showed to contain 2000 Arbitarary Units (AU).

The bacteriocin plantaricin UG1 was prepared according to the procedures described previously (Enan, 2002, 2005). Briefly, a 16 h old culture in MRS broth of *Lactobacillus plantarum* UG1 was centrifuged at 10000×g for 10 min at 4°C. Cell free supernatants were subjected to ammonium sulphate precipitation. The precipitates were recovered in 10mM potassium phosphate buffer pH 6.5 and were sterilized by filtration (0.22 μm Milipore). One milliliter of partially purified plantaricin UG1 was assayed against *Listeria monocytogenes* LMG10470 and was showed to contain 1920 Arbitarary Units (AU).

Inhibition of *L. monocytogenes* LMG10470 by bacteriocins *in vitro* and in food: The food samples mentioned above were inoculated with 10^5 cfu mL^{-1} or gm final concentration. The above inoculated food samples were treated with either 4000 AU mL^{-1} of nisin or 4000 AU mL^{-1} plantaricin UG1. These bacteriocins were used individually or in combination. The inoculated and treated samples were shaken vigorously by hand to distribute both inocula and bacteriocins and were then incubated at 8°C for 4 days. The survivors of listerias cells were enumerated onto *Listeria* selective agar at different time intervals. Each test was run in duplicate and mean values were calculated. The above procedure was based on the procedure proposed by Vignolo *et al.* (2000). The regrowing resistant variants as well as resistance frequency were studied by Enan (2005).

RESULTS

The effect of nisin and plantaricin UG1 individually or in combination on the growth of the *L. monocytogenes* LMG10470 cells in BHI broth and in pasteurized milk is shown in Fig. 1. The action of nisin and plantaricin UG1 was characterized by a two phase response: at first, viable listerias populations decreased by 2-3 log cycles within almost 36-48 h in either BHI broth or pasteurized milk. However, no growth was observed in all samples tested after almost 3 days. Thereafter, a resurgence of growth was observed. The mix of nisin and plantaricin UG1 prevented regrowth of the *L. monocytogenes* LMG10470 cells in both BHI broth and pasteurized milk.

Figure 2 shows the effect of bacteriocins on growth of the *L. monocytogenes* LMG10470 strain in tomato paste. Nisin-plantaricin UG1 mixture displayed the fastest rate of bactericidal action recording 100% cell destruction within 12 h, followed by both nisin and plantaricin UG1 which destroyed 100% of listerias cells within 48-60 h. By further storage of the treated food samples, no regrowth was observed in tomato paste samples treated with nisin-plantaricin UG1 mixture, but a slight regrowth (around 1×10^2) was observed after one week in tomato paste samples treated with either nisin or plantaricin UG1.

The addition of nisin; plantaricin UG1; nisin-plantaricin UG1 mixture to either minced meat or kareesh cheese samples (Fig. 3) resulted in a marked bactericidal effect with reduction in viable counts of *L. monocytogenes* LMG10740 by 3 log cycles; 3 log cycles; 5 log cycles after almost 48-60 h in minced meat, respectively and by 3 log cycles; 2 log cycles; 5 log cycles after almost 48-60 h in kareesh cheese samples (Fig. 3). After three to four days, no growth of listerias cells was observed in the treated samples of minced meat and Kareesh cheese; but thereafter a slight regrowth was observed only in samples treated with either nisin or plantaricin UG1. However, no regrowth was observed in samples of both minced meat and Kareesh cheese treated with nisin-plantaricin UG1 mixture (Fig. 3).

A representatives of the survived cells of *L. monocytogenes* LMG10470 were isolated from all food samples treated with either nisin or plantaricin UG1 and were proved to be resistant variants as they were still resistant to the respective bacteriocins onto BHI agar

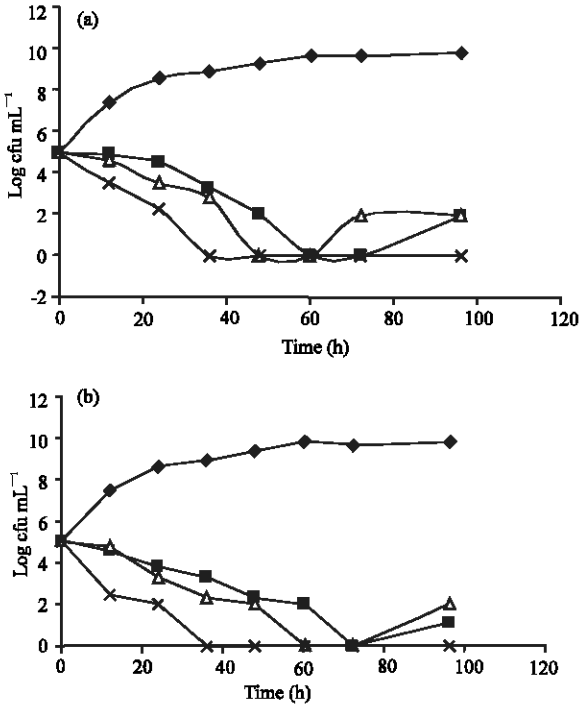

Fig. 1: Growth of *Listeria monocytogenes* LMG 10470 in (a) brain heart infusion broth and in (b) pasteurized milk held at 8°C with or without bacteriocins. (◆) control without bacterovin; (■), (▲) and (✕), in presence of nisin, plantaricin UGI and nisin-plantaricin UGI mixture, respectively

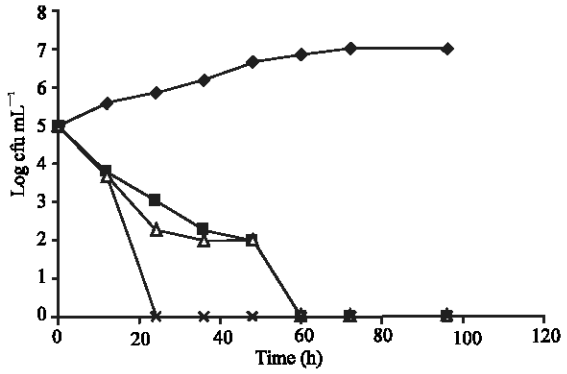

Fig. 2: Growth of *Listeria monocytogenes* LMG 10470 in tomato paste held at 8°C with or without bacteriocins. (◆) control without bacterovin; (■), (▲) and (✕), in presence of nisin, plantaricin UGI and nisin-plantaricin UGI mixture, respectively

plates after 7 successive transfers. Mutants of listerias cells resistant to either nisin or plantaricin UG1 showed a

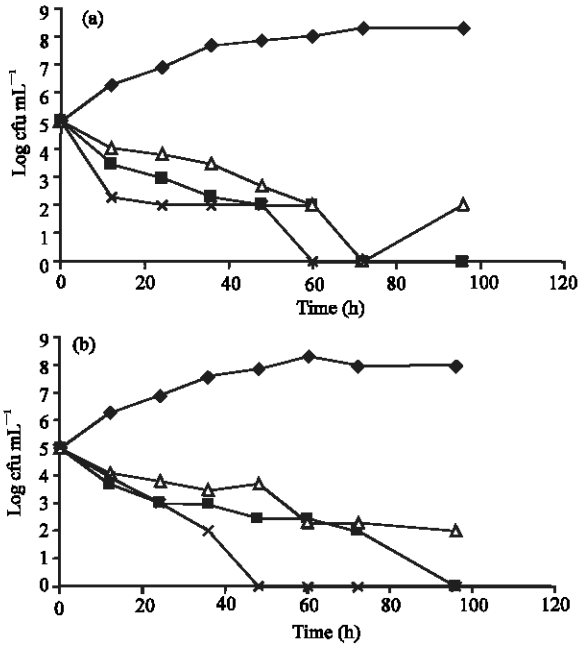

Fig. 3: Growth of *Listeria monocytogenes* LMG 10470 in (a) minced meat and in (b) kareesh cheese held at 8°C with or without bacteriocins. (◆) control without bacterovin; (■), (▲) and (✕), in presence of nisin, plantaricin UGI and nisin-plantaricin UGI mixture, respectively

resistance frequency in the range of 10^{-5} to 10^{-6}. When resistance frequency in the range of 10^{-5} to 10^{-6}. When the sensitivity of the mutant listerias cells to the respective bacteriocins in media containing either nisin or plantericin UG1 was tested, the variants didn't show cross-resistance. The survivors isolated from samples containing nisin were resistant only to nisin but were sensitive to plantericin UG1. Also, the plantaricin UG1 mutants were sensitive to nisin and where insensitive to this bacteriocin.

DISCUSSION

Bacteriocins of lactic acid bacteria, natural antimicrobial peptides, have been proposed by many researchers for controlling *L. monocytogenes* in food products (Muriana, 1996). Nisin produced by some *Lactococcus lactis* subsp. *Lactis* strains, belongs to class I bacteriocins (lantibiotic peptides) (Klaenhammer, 1993). This bacteriocin has been approved by the World Health Organization as a preservatique in the food industry (Ronk, 1988). Unfortunately, a spontaneous resistant variants of *L. monocytogenes* to nisin were detected and characterized Rekhif *et al*. (1994), Mazzotta and Montville

(1997) and Vignolo *et al*. (2000). This clearly suggests that there is a need to continue research to explain the nature of resistance and to find out other bacteriocins having different modes of resistance to be mixed with nisin.

The effect of either nisin or plantaricin UG1 noticed in this study induced a bactericidal action on *L. monocytogenes* followed by regrowth of the target cells. This transitory inhibitory effect has been reported previously for nisin (Bouttefroy and Milliere, 2000) and plantaricin UG1 (Enan, 2002). Representatives of the regrowing cells were isolated and were proved to be resistant cells as they were still resistant to either nisin or plantaricin UG1 in BHI agar containing the same bacteriocins. The resistant variants of *L. monocytogenes* resistant to either nisin (Vignolo *et al*., 2000) or plantaricin UG1 (Enan, 2005) had been isolated and characterized. The results obtained have showed that the resistance frequency in *L. monocytogenes* to either nisin or plantaricin UG1 was showed to be 10^{-5} to 10^{-6}. Many authors also (Davis and Adams, 1994; Mazzota and Montville, 1997) showed that nsin resistant variants of *L. monocytogenes* appear at frequencies of 10^{-5} to 10^{-8}. Also, Enan (2005) showed a plantericin UG1 resistance in *L. monocytogenes* at a resistance frequency around 10^{-6}. Nisin resistance response of *L. monocytogenes* was reported to be correlated with membrane fatty acid composition, a significant reduction in membranes phospholipids and, therefore, inability of nisin to form pores in more rigid membranes (Abee, 1995). In contrast the resistance phenotype of listerias cells to plantaricin UG1 was due to modification in adsorption site (s) of the target cells (Enan *et al*., 1996). A combination of two bacteriiocins differying in their modes of resistance have a higher protective ability for foods against *L. monocytogenes* strains than when used individually (Hanlin *et al*., 1993).

The results presented here indicate that nisin-plantaricin UG1 combinations avoided the regrowth of bacteriocin resistant cells of *L. monocytogenes in vitro* and in foods (pasteurized milk, tomato paste, minced meat, kareesh cheese). Such results supported many published results in this respect (Hanlin *et al*., 1993; Bouttefroy and Milliere, 2000; Vignolo *et al*., 2000). A mixture of two bacteriocins would be more bactericidal than one bacteriocin, since cells resistant to one bacteriocin would be killed by another. In addition mainly synergistic effects were reported when the interactions between pair of bacteriocins were used (Mulet-Powell *et al*., 1998). Moreever, a mixture containing two bacteriocins that belong to different classes would be more effective in food protection than one only. This is the case in this study, nisin is a lantibiotic (Moll *et al*., 1999) (Class I). However, plantaricin UG1 is probably class IIb bacteriocins (Enan *et al*., 2004). No resistant variants of *L. monocytogenes* were isolated from foods samples treated with nisin-plantaricin UG1mixture. This is a promising result to use nisin-plantaricin UG1 mixture as biopreservative for dairy products, tomato paste and minced products, tomato paste and minced meat in Saudi Arabia. No cross resistance was found between nisin and plantaricin UG1 whose synergistic listericidal effect prevented the emergence of spontaneous variants of *L. monocytogenes* in pasteurized milk, tomato paste kareesh cheese and minced meet.

Based on the results employed herein and in agreement with Vignolo *et al*. (2000), the industrial application of combined partically purified and/or purified bacteriocins in food products (dairy products, tomato paste and minced meat could be a useful approach to reduce the frequency at which resistant populations develop, to improve the hygiene standards and to extend the shelf life of dairy products, meat products and tomato paste. Although the use of nisin is permitted in more than 50 countries in a variety of foods, other bacteriocins with different and/or more effective antibacterial activity may be considered and approved to mix with nisin to be of more hazardous quality.

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