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Synergistic effect of nisin and garlic shoot juice against *Listeria monocytogenes* in milk

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Abstract

The aim of this research was to determine the synergistic effect of nisin and garlic shoot juice (GSJ) against *Listeria monocytogenes* ATCC 19118 found in whole (3.5%), low (1%) and skim (no fat content) milk. Garlic shoot juice (GSJ) at concentrations of 2.5%, 5% and 10% revealed strong and similar patterns of antilisterial effect against *L. monocytogenes* ATCC 19118 in all categories of milk. Nisin only at concentrations of 62.5, 125, 250 and 500 IU/ml displayed a strong antilisterial effect as compared to the control group. Also, the synergistic combinations of GSJ (2.5%, 5%) and nisin (62.5, 125, 250 and 500 IU/ml) had a remarkable antilisterial activity in all categories of whole, low and skim milk after 14 days. Results of this study indicated the synergistic effect of GSJ and nisin as a potential antilisterial agent for the food industry.

Keywords: Synergistic effect; Antilisterial activity; Garlic shoot juice (GSJ); Nisin; *Listeria monocytogenes* ATCC 19118

1. Introduction

Listeria monocytogenes has been implicated in several food-borne outbreaks associated with the consumption of pasteurized milk (Fleming et al., 1985). The pathogen can cause bovine mastitis and is occasionally found in raw milk (Liewen & Plautz, 1988). The Food and Drug Administration (FDA) reported that 5% of raw milk samples tested was found positive for *Listeria*, and 18% of the milk from lactating Holstein cows contained *L. monocytogenes* serotype 4b. Raw milk was the source of the first case of food-borne listeriosis reported in 1953 in Germany, and the first listeriosis outbreak associated with milk in the United States occurred in 1979 (Ryser, 1999). Although *L. monocytogenes* is destroyed by pasteurization, several studies have reported its heat resistance and its ability to survive pasteurization due, in part, to the protective nature of the leukocyte in which the pathogen may be present (Doyle et al., 1987).

L. monocytogenes has the ability to grow at temperatures ranging from 1 to 45 °C (George, Lund, & Brocklehurst, 1988). It also grows at higher rates due to temperature abuses which may be encountered during warehouse storage, transportation, retail display, consumer transportation and consumer storage at home. A variety of different chemical and synthetic compounds have been used as antimicrobials to inhibit *L. monocytogenes* in food systems. It is very important to inhibit the growth of such microorganisms that cause decay and transmutation in stored food. Currently there are conflicting opinions concerning the possible heat resistance of *L. monocytogenes*. Some have reported that *L. monocytogenes* is more thermotolerant than most nonspore-forming bacterial pathogens and may in some instances survive at minimum pasteurization treatment, as supported by the findings of others (Garayzabal, Rodriguez, Boland, Cancelo, & Fernandez, 1986). Recently, consumers are concerned about the safety of foods containing preservatives. Therefore, antimicrobial substances from

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natural sources like plants have been investigated to achieve higher levels of food safety standards (Hao, Brackett, & Doyle, 1998).

Garlic is a widely distributed plant used in all parts of the world not only as a spice but also as a popular remedy for several diseases. A wide range of microorganisms including bacteria, fungi, protozoa and viruses have been shown to be sensitive to crushed garlic preparations (Cavallito, Bailey, & Suter, 1944; Delaha & Garagusi, 1985; Ghannoum, 1988). Moreover, garlic has been reported to reduce blood lipid and cholesterol levels (Gebhardt & Beck, 1996), possess anticancer effects and prevent aging (Hong, Ham, Choi, & Kim, 2000; Sheen, Li, Sheu, Meng, & Tsai, 1996). The analysis of nutritional and volatile flavor compounds of garlic shoots has already been reported (Kim & Chung, 1997), but there are few reports that have been published so far on the biological effect of garlic shoots. Indeed, it is necessary to study the potential applications of garlic shoot juice, a by-product of garlic.

Nisin is a well-known broad spectrum bacteriocin active against Gram-positive pathogens associated with foods. Its use as a food biopreservative is limited by the lack of effect against Gram-negative bacteria; moreover, the development of nisin resistance has been reported in sensitive Gram-positive pathogens (Ming & Daeschel, 1993). The combination of bacteriocins with other preservation mechanisms has been reported to reduce the selection for resistance to bacteriocins in target strains or to extend its inhibitory activity to Gram-negative species (Stevens, Sheldon, Klapes, & Klaenhammer, 1991). Recent studies have shown that the spectrum of activity of nisin may also be extended to Gram-negative bacteria by using it in combination with other agents (Cutter & Siragusa, 1995). Many reports have been published on the synergistic antimicrobial effects of nisin with sucrose fatty acid esters (Thomas, Davies, Delves-Broughton, & Wimpenny, 1998), the lactoperoxidase system (Boussouel et al., 1999), thymol (Ettayebi, Yamani, & Rossi-Hassani, 2000) and carbon dioxide (Nilsson et al., 2000). Therefore, this study was performed to determine the synergistic effect of nisin and garlic shoot juice against *L. monocytogenes* present in the varied categories of whole, low and skim milk, as a potential preservative for use in the food industry.

2. Materials and methods

2.1. Preparation of garlic shoot juice (GSJ)

Garlic shoots were purchased from a local market in Gyungsan City, Korea. The samples were prepared by our method (Kim, Choi, & Kang, 2007). The collected samples of garlic shoot were washed in running water followed by rinsing in distilled water. After the removal of water content from garlic shoot samples by using sterilized gauge, the garlic shoots were finally dried at room temperature for 2–3 h. For sample preparation, the garlic shoot samples were mixed with distilled water at the ratio of 1:1 (w/v), then ground by using a mixer (ARTLON, Model DA282-2, Korea); 50% (w/v) juice of garlic shoot (GSJ) was obtained. The product was centrifuged at 18,000 rpm for 30 min at 4 °C. The supernatant of garlic shoot juice was collected and passed through a 0.45 μm filter (Millipore Co., USA), and stored at 4 °C until further analysis.

2.2. Chemicals

Nisin was purchased from Sigma Chemical Co. (N5764; Sigma, St. Louis, Missouri, USA). Nisin stock solution was prepared with 0.02 N HCl (6.25×10^4 IU/ml) and was sterilized by autoclaving at 121 °C for 15 min, and kept in a refrigerator at 4 °C until used.

2.3. Preparation of milk samples

Pasteurized and homogenized grade A milk samples consisting of whole (3.5% fat), low (1% fat) and skim milk (no fat) were obtained from a local market.

2.4. Preparation and maintenance of L. monocytogenes

The strain *L. monocytogenes* ATCC 19118 used in this study was collected from the Korean Agricultural Culture Collection, Suwon, Republic of Korea. The strain was maintained on BHI agar (brain heart infusion, Difco) at 4 °C and was grown in BHI broth at 37 °C for 24 h.

2.5. Inhibitory effect of garlic shoot juice (GSJ)

The varied concentrations of garlic shoot juice (GSJ) (2.5%, 5% and 10%) were added to whole, low and skim milk and inoculated with initial population of approximately 2×10^4 cfu/ml of *Listeria* strain ATCC 19118, respectively. Controls were inoculated with *Listeria* strain without GSJ, and stored at 0, 2, 4, 6, 8, 10, 12 and 14 days at 4 °C. The colonies were counted in NA (nutrient agar, Difco) at 37 °C after 24 h of incubation.

2.6. Inhibitory effect of nisin

To determine the inhibitory effect of nisin, varied concentrations of nisin (62.5, 125, 250 and 500 IU/ml) were added to whole, low and skim milk samples and were inoculated with an initial population of approximately 2×10^4 cfu/ml of *Listeria* strain ATCC 19118, respectively. Controls were inoculated with *Listeria* strain without nisin, and stored at 0, 2, 4, 6, 8, 10, 12 and 14 days at 4 °C. The colonies were counted in NA (nutrient agar, Difco) at 37 °C after 24 h of incubation.

2.7. Synergistic effect by GSJ and nisin

To determine the synergistic effect of garlic shoot juice (GSJ) and nisin, two concentrations of GSJ (2.5% and 5%) in combination with 62.5, 125, 250 and 500 IU/ml of nisin were added to whole, low and skim milk, and inoculated with initial population of approximately 2×10^4 cfu/ml of *Listeria* strain ATCC 19118, respectively. Controls were inoculated with *Listeria* strain without GSJ and nisin, and stored at 0, 2, 4, 6, 8, 10, 12 and 14 days at 4 °C. The colonies were counted in NA (nutrient agar, Difco) at 37 °C after 24 h of incubation.

2.8. Data analysis

Each experiment was run in triplicate, and the mean values were calculated. The counters were expressed as log cfu/ml. Comparison of means was performed using Duncan's multiple test with a significance level of $\alpha = 0.05$.

3. Results

3.1. Antilisterial activity of GSJ in milk

It was observed that a similar high antilisterial activity of GSJ (2.5%, 5% and 10%) was found at the used concentrations against *L. monocytogenes* ATCC 19118 as compared to the control (Fig. 1). The initial population of *L. monocytogenes* in whole, low and skim milk was 4.32 log cfu/ml. Controls were counted at 6.36, 6.61 and 7.17 log cfu/ml for 8 days, respectively. In the case of whole milk, 2.5%, 5% and 10% GSJ showed the difference of 2.29, 2.53 and 2.75 log cfu/ml, respectively, after 8 days as compared to control groups. Whereas, in low milk, colony count differences were found as 2.43, 2.81 and 3.25 log cfu/ml as compared to control after 8 days. For skim milk, colony count differences were observed as 2.93, 3.35 and 3.58 log cfu/ml, respectively, after 8 days. Ten percent GSJ gave a remarkable decrease in the growth of *L. monocytogenes* ATCC 19118 at day 12, and inhibited the cell population by about 4.00–4.50 log cfu/ml as compared to control at day 14 in all milk samples.

3.2. Antilisterial activity of nisin in milk

It was found that nisin at the used concentrations of 62.5, 125, 250 and 500 IU/ml showed strong antilisterial effect against the growth of *L. monocytogenes* in low fat and skim milk as compared to control groups (Fig. 2). Whereas, moderate antilisterial activity was observed in whole milk. The controls were counted at 7.42, 7.56 and 7.57 log cfu/ml after 14 days. In all treated group, the initial cell number was rapidly decreased for 2 days. In the case of skim milk, nisin at 250 and 500 IU/ml led to a drastic decline in the initial cell count numbers for 2 days and no cell growth was observed until 10 days. It was found that the decrease in cell count numbers of *L. monocytogenes* was dependent on fat contents present in whole, low fat and skim milk.

3.3. Synergistic effect on the antilisterial activity of GSJ and nisin in milk

It was observed that GSJ (2.5% and 5%) in combination with different concentrations of nisin (62.5, 125, 250 and

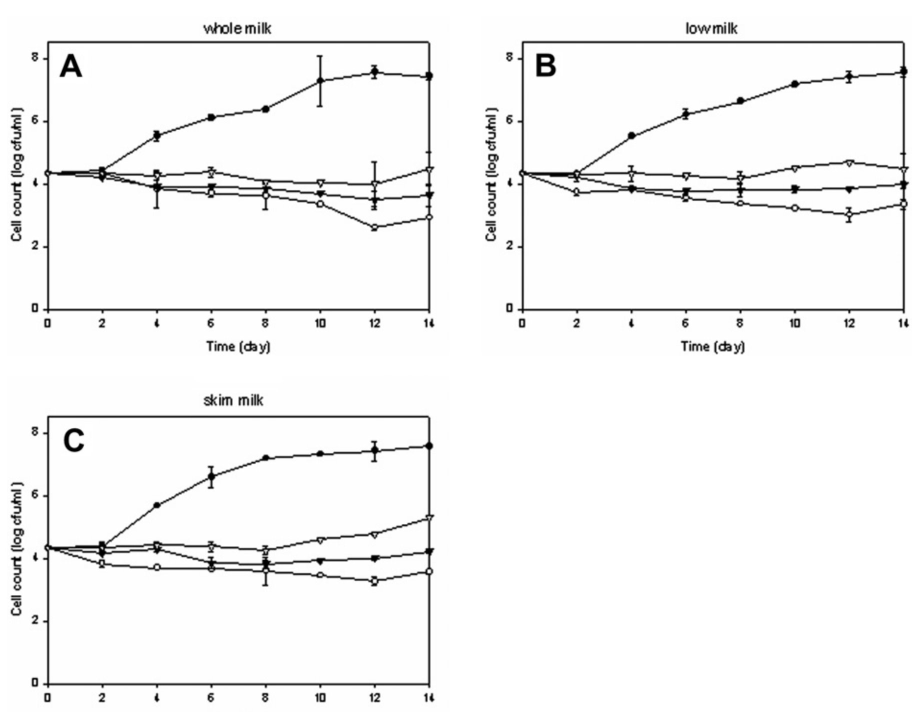

Fig. 1. Inhibitory effect of GSJ against *Listeria monocytogenes* ATCC 19118 in whole (A), low (B) and skim (C) milk for 14 days. ●, control; ▽, 2.5% GSJ; ▼, 5% GSJ; ○, 10% GSJ.

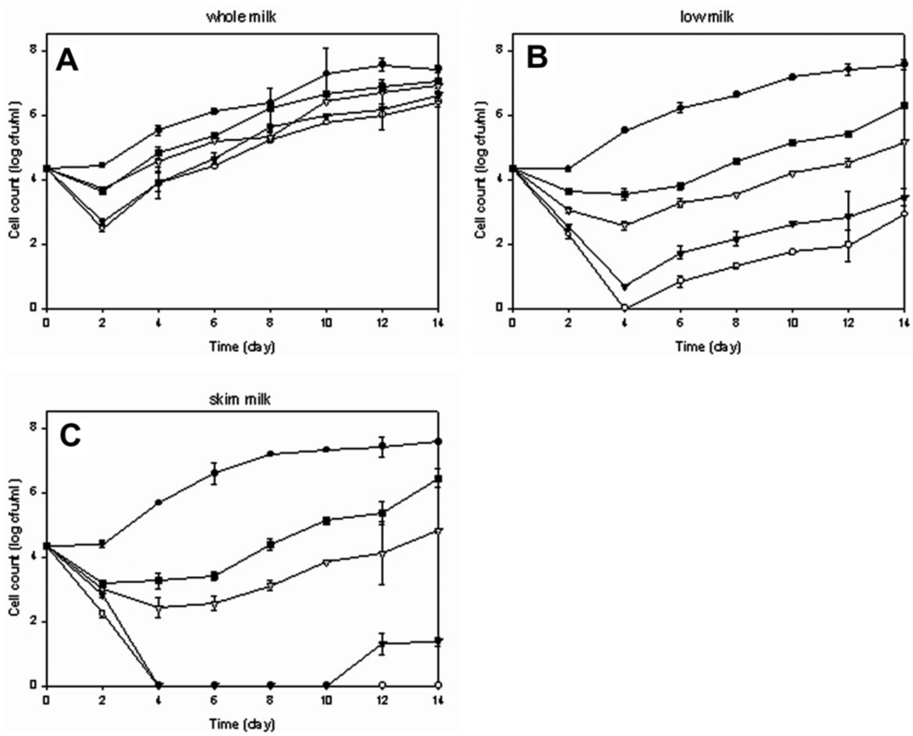

Fig. 2. Inhibitory effect of nisin against *Listeria monocytogenes* ATCC 19118 in whole (A), low (B) and skim (C) milk for 14 days. ●, control; ■, nisin 62.5; ▽, nisin 125; ▼, nisin 250; ○, nisin 500 IU/ml.

500 IU/ml) had a potential antilisterial activity with a synergistic effect in whole, low fat and skim milk at day 14. A strong antilisterial effect was confirmed when milk samples were treated with 5% GSJ in combination with various concentrations of nisin as compared to 2.5% GSJ. In tests with whole milk, the combined groups had a stronger inhibitory ability against *L. monocytogenes* ATCC 19118 as compared to the control (Fig. 3). Both 2.5% and 5% GSJ and 62.5 IU/ml nisin as controls were found to display differences in antilisterial effect as compared to the negative control with their respective cell count values of 2.94, 3.78 and 0.37 log cfu/ml. However, the combined action of 2.5% GSJ with various concentrations of nisin (62.5, 125, 250 and 500 IU/ml) essentially decreased the cell count numbers in whole milk. As shown in Fig. 4, the addition of 2.5% and 5% GSJ alone to the low milk reduced cell count numbers about 3.09 and 3.58 log cfu/ml, respectively as compared to the control. The addition of nisin 62.5 IU/ml alone had little antilisterial effect as compared to 2.5% and 5% GSJ alone. Whereas, the combined groups of

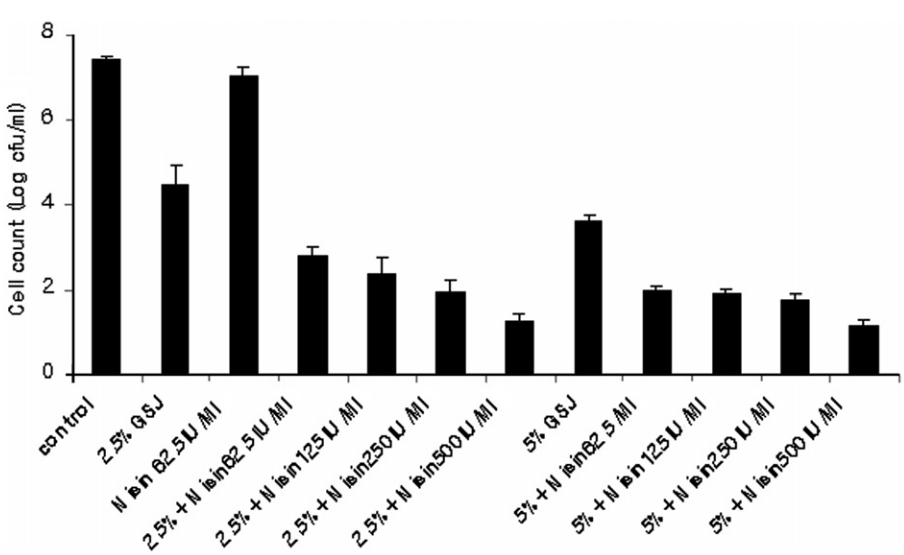

Fig. 3. Synergistic effect of the various combinations of GSJ and nisin on *Listeria monocytogenes* ATCC 19118 in whole milk at 14 days.

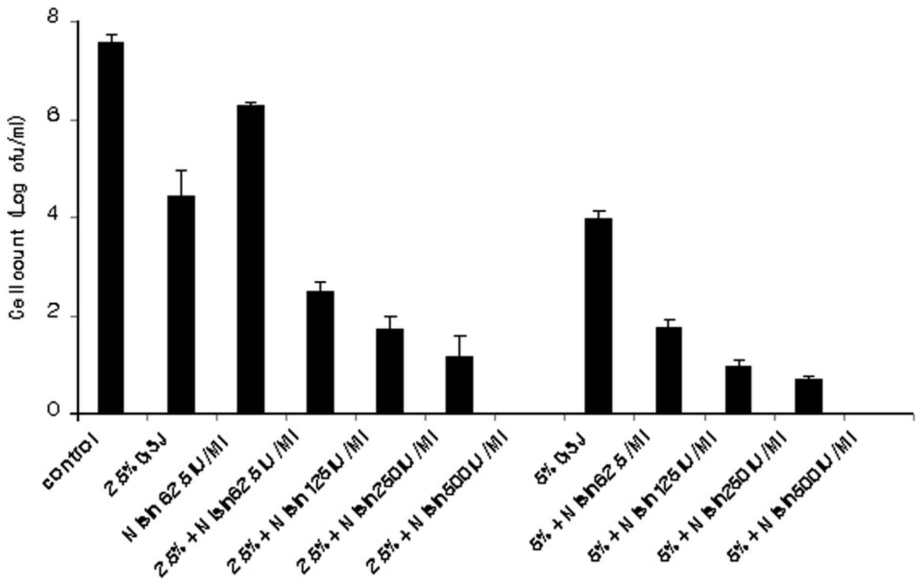

Fig. 4. Synergistic effect of the various combinations of GSJ and nisin on *Listeria monocytogenes* ATCC 19118 in low milk at 14 days.

GSJ and nisin had a potent antilisterial effect in low fat milk, especially GSJ (2.5% and 5%) in combination with 500 IU/ml nisin completely inhibited the growth of *L. monocytogenes* ATCC 19118, with no cell count observed at 14 day. As for the results represented in Fig. 5 for the combined effect of GSJ and nisin, the combined group of GSJ (2.5%) and nisin (500 IU/ml) produced a great reduction in the growth of *L. monocytogenes* ATCC 19118 in skim milk as indicated by the complete inhibition of cell count numbers. However, 5% GSJ along with nisin (62.5, 125, 250 and 500 IU/ml) revealed desirable antilisterial activity, representing complete inhibition of the *L. monocytogenes* strain at all combinations in skim milk. Other combinations of 2.5% GSJ and nisin (62.5, 125 and 250 IU/ml) also brought about a combined antilisterial effect.

4. Discussion

The emergence of *L. monocytogenes* as an important food-borne pathogen has led to a resurgence of interest in antimicrobials suitable for its control. At the same time, consumer demand for foods that contain fewer preservatives, are less processed, free from artificial additives and perceived as fresh and more natural food stuffs has increased (Gould, 1992). To address both these issues, much research has focused on the potential of garlic shoot, a by-product of garlic for its use in food preservation. In the present study, it was found that garlic shoot juice with various combinations of nisin has a great influence as an antilisterial effect in whole, low fat and skim milk.

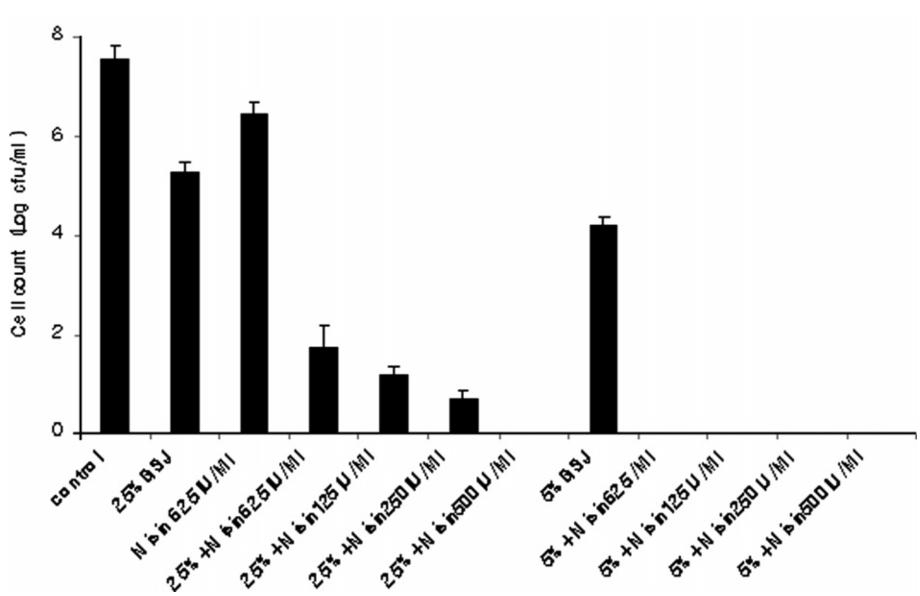

Fig. 5. Synergistic effect of the various combinations of GSI and nisin on *Listeria monocytogenes* ATCC 19118 in skim milk at 14 days.

In experiments to assess GSJ activity, a potential antilisterial effect was revealed at the used concentrations at day 4 in whole, low fat and skim milk as compared to control groups. In particular, 10% GSJ displayed strong antilisterial activity with no dependency of fat content present in all categories of milk. Others have also reported that garlic extract has remarkable ability to inhibit several food-borne pathogens including *L. monocytogenes* (Saleem & Al-delaimy, 1982; Wills, 1956). These finding were confirmed in our previous study that garlic shoot juice (GSJ) was found to have severe detrimental effect on the growth and morphology of *L. monocytogenes* (Kim et al., 2007). In the results reported here, it has been found that GSJ has a strong antilisterial effect through the direct growth inhibition of *L. monocytogenes* in whole, low and skim milk. As shown in Fig. 2, the addition of nisin in whole, low fat and skim milk displayed strong antilisterial effect at the used concentrations of 62.5, 125, 250 and 500 IU/ml. Strong antilisterial effect of nisin was observed in low fat and skim milk, whereas, in whole milk there was only a slight susceptibility to the used concentrations of nisin as compared to the control. These findings are strongly supported by the work of other researchers as they also observed that nisin alone was found more effective in controlling food-borne pathogens in skim milk than in whole milk, which might be due to the different fat contents (Jones, 1974).

Jung, Bodyfelt, and Daeschel (1992) reported that the activity of nisin against *L. monocytogenes* in fluid milk was directly dependent upon the fat contents. These findings are strongly supported by the work of Meena, Aparna, and Leora (2004), as they also observed that *L. monocytogenes* was found most sensitive to nisin in skim milk, which showed a rapid decline in the cell numbers up to <10 cfu/ml after 12 days. However, an initial decline in cell number of *L. monocytogenes* as an antilisterial efficacy of nisin in whole milk was found at 2%.

As shown in Fig. 3, the effect of nisin (62.5, 125, 250 and 500 IU/ml) was found to be enhanced by a combined action in whole milk with 2.5% and 5% GSJ. The pathogen cell count was decreased up to 6.11 and 6.24 cfu/ml by the combined effect of each of 2.5% and 5% GSJ, and 500 IU/ml nisin, respectively, in whole milk. The combined effect of 2.5% GSJ and 62.5 IU/ml nisin showed a reduction of *L. monocytegones* of about 4.61 log cfu/ml (50%) in whole milk, whereas, 5% GSJ and 62.5 IU/ml nisin showed about 5.4 log cfu/ml cell count decrease. Other combined groups also exhibited strong antilisterial activity as compared to the control. The synergistic effect of 5% GSJ and 500 IU/ml nisin showed the antilisterial effect as a remarkable cell count decrease of 6.24 log cfu/ml.

In tests with low fat milk, 2.5% and 5% GSJ along with 500 IU/ml nisin drastically decreased the cell count numbers, and no cell growth of *L. monocytogenes* was observed. Also nisin at the varied concentrations of 62.5, 125 and 250 IU/ml with 2.5% and 5% GSJ exhibited strong antilisterial effect in low fat milk as compared to control groups. As the results displayed in Fig. 5 the combinations of GSJ (2.5%) along with nisin (62.5, 125, 250 and 500 IU/ml) synergistically reduced the cell count numbers of *L. monocytogenes* ranging from 5.8 to 7.6 cfu/ml in skim milk, whereas, 5% GSJ along with all the used concentrations of nisin (62.5, 125, 250 and 500 IU/ml) completely inhibited the growth of *L. monocytogenes* in skim milk with no cell count observation. The combinations of 2.5% and 5% GSJ along with 500 IU/ml nisin had a very strong synergistic effect against *L. monocytogenes* in whole, low fat and skim milk as compared to control. Based on the above results, it is confirmed that GSJ and nisin have a strong synergistic effect as an antlisterial potential against *L. monocytogenes*.

The results of Bhurinder, Falahee, & Adams, 2001 support our findings that GSJ and nisin may act synergistically against *Listerial* strains. Indeed, a literature survey has revealed that food preservation by multiple preservatives in a small amount may be counted as superior to a large amount of a single preservative in order to secure both microbial stability and safety to maintain the sensory, nutritive and economic properties of the foods (Bhurinder et al., 2001; Leistner & Gorrism, 1995). Thus, such synergistic combinations of GSJ and nisin could become the alternative industrial products to synthetic bactericides for use in food industries.

Recently, the synergistic effect of the nisin with various essential oils has been conclusively demonstrated and it has been noted that the activity of the essential oil constituents such as carvacrol and thymol is enhanced by the presence of nisin (Blaszyk & Holley, 1998; Ettayebi et al., 2000; Pol & Smid, 1999). In this study, we determined an additive effect of the two preservatives as the antilisterial potential to test the synergistic effect of various combinations of GSJ and nisin. However, several reports have been published on the antibacterial effect of nisin with various extracts but no report has been found or published in the literature on the synergistic effect of GSJ and nisin against *L. monocytogenes* in whole, low fat and skim milk.

Henning, Metz, and Hammes (1986) proposed that the reaction between nisin and the listerial cell membrane was caused by hydrophobic interaction between the amino acid residues of nisin and the fatty acids of the membrane phospholipids. It was further suggested that the electrostatic attraction between nisin molecules and the negatively charged phospholipids is involved in the antilisterial effect (Sahl & Bierbaum, 1998). Ming and Daeschel (1995) compared the sensitivity to nisin of cells of two strains of *L. monocytogenes* Scott A: one with significantly decreased phospholipid content compared to the parental strain (31 vs. 46 mg/g, dry weight). They observed low antilisterial activity of nisin in the strain with the low phospholipid content. The nisin resistant cells were found to bind less nisin and release less phospholipid than the sensitive cells when treated with the same concentrations of nisin. The authors attributed the effect to the decreased phospholipid component of the membrane of the resistant cells, and concluded that nisin reacts with the phospholipids, and that cellular damage depends on the amount of membrane phospholipid present (Swaisgood, 1985). Meena et al (2004) reported that the phospholipids present in 2% fat market milk that is pasteurized and homogenized appear to bind a large portion of the added nisin, resulting in a reduced nisin available to react with the cell membrane of *L. monocytogenes*, and thereby a reduced antilisterial activity. This was not the case in skim milk, where similar nisin concentrations were sufficient to cause disruption of the listerial cell membrane. Also maximum antilisterial effects of nisin in skim milk and reduced effects in milk with 2% and 3.5% fat or higher were obtained. These results strongly support the findings of this present investigation.

In our study, we found that GSJ and nisin synergistically and significantly inhibited the growth of *L. monoctogenes* in all categories of the milk. Thus, the results of the study indicate that such combinations of GSJ and nisin have the significant values to apply in milk industries as potential food preservatives.

Acknowledgement

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