Antagonist effect of *Enterococcus durans* E204 isolated from camel milk of Morocco, against *Listeria monocytogens* CECT 4032 in skimmed milk

El Ouardy KHAY*. Mohamed BENSBIH. Naima EL MOUSSAOUI. Mohamed IDAOMAR. Jamal ABRINI

Equipe de Biotechnologies et Microbiologie Appliquée, Laboratoire de Biologie et Santé, Département de Biologie, Faculté des Sciences, Université Abdelmalek Essaâdi, BP 2121, 93002 Tétouan, Morocco
*Corresponding author: kelouardy@yahoo.fr

Abstract: This study investigated the antibacterial activity of *Enterococcus durans* E204, isolated from camel milk of Morocco, against *Listeria monocytogenes* CECT 4032. The growth and the production of bacteriocin-like inhibitory substance (BLIS) by *E. durans* E204 were evaluated in De Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostic) as well as in skimmed milk (SM) obtained from a local market. When inoculated alone, the target strain (10^6 CFU/ml) grew very well in SM reaching a maximum cell number of 11.8 log CFU/ml after 12 hours of incubation. However, when exposed to *E. durans* (10^8 log CFU/ml), *L. monocytogenes* was totally eliminated after 16 hours of incubation. The pH of SM declined steadily throughout the fermentation period and was approximately 4.2 after 48 hours of co-culture. In addition, When the cell free supernatant of mono-culture of the producer strain was added (at 10, 50 and 100 ml volume) at the beginning of growth of *L. monocytogenes* at both concentrations (10^6 and 10^4 CFU/ml), rapid deceases of cell number were observed in the first 4 hours of incubation and thereafter the pathogen regained a steady growth. Further experiment showed that BLIS exhibits a bactericidal effect against *L. monocytogenes* in Brain Heart Infusion (BHI) broth since it caused depletion on its optical density. Our results suggest that *E. durans* E204 with antilisterial activity could be used as a protective culture to enhance the safety of dairy products.

[El Ouardy KHAY, Mohamed BENSBIH, Naima EL MOUSSAOUI, Mohamed IDAOMAR, Jamal ABRINI. **Antagonist effect of *Enterococcus durans* E204 isolated from camel milk of Morocco, against *Listeria monocytogens* CECT 4032 in skimmed milk.** *Life Sci J* 2013; 10(2): 2678-2683]. (ISSN: 1097-8135). http://www.lifesciencesite.com 371

Keywords: *Enterococcus durans*, *Listeria monocytogenes*, co-culture, skimmed milk, bactericidal effect

1. Introduction

Listeria monocytogenes can grow and multiply under harsh conditions which explains its ubiquity. It is mainly isolated from raw milk, dairy products and dairy plants (Farber and Peterkin 1991; Mahmoodi 2010; Mugampoza et al.2011) and it is the agent of listeriosis a serious disease for humans. Raw milk is generally contaminated by *L. monocytogenes*, mainly due to sick animals in the farm. The main source of animal contamination by *L. monocytogenes* is the poor quality of silage preparation (Fenlon 1986). In dairy industry, post-pasteurization contamination occurs during processing (Vogel et al. 2001). *Listeria monocytogenes* can survive during storage at low temperatures and on process equipment with ability to act as constant source of contamination (Pan et al. 2006).

Dairy products have been frequently contaminated and associated with listeriosis outbreaks (Rocourt 1996). Hence, control of *L. monocytogenes* in milk, cheese and other dairy products has been largely studied (Piccini and Shelef 1995; Davies et al.1997; Wan et al.1997; Rodriguez et al.1997; McAuliffe et al.1999; Benkerroum et al. 2000). Protection against listeriosis outbreaks in the food manufacturing must focuses on all production stages, starting from milk production in the farm, in dairy plant, during storage and distribution. *Listeria monocytogenes* survive longer in skimmed milk with high content of dry matter and high pH value compared to full fat milk (Griffit and Deibel 1990).

To prevent the survival and growth of *Listeria*, several studies have demonstrated the antagonistic activity of autochthonous microflora isolated from dairy products against *L. monocytogenes* (Coventry et al.1997; Ortolani et al. 2010). For instance, *E. durans* E204, a strain isolated from camel milk of Morocco, is a producer of bacteriocin-like inhibitory substance (Khay et al.2011; 2012). In this study, the ability of *E. durans* E204 to antagonize *L. monocytogenes* during co-cultivation in skimmed milk was assessed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Listeria monocytogenes CECT 4032, a pathogenic strain was obtained from Spanish Type Culture Collection (Colección Española de Cultivos Typo, CECT). *Enterococcus durans* E204 was isolated from camel milk of Morocco. Bacterial strains were stored at - 80°C in their growth medium containing 35% (v/v) glycerol. For challenge tests, *L.*

monocytogenes and *E. durans* E204 were sub-cultured twice successively in Brain Heart Infusion (BHI) (Biokar Diagnostics, Beauvais, France) and in MRS broth (Biokar Diagnostics, Beauvais, France) respectively, for 24 h at 30°C. Cultures were then diluted in sterile peptone saline buffer (0.1% peptone and 0.85% NaCl, pH 7.0) to obtain the concentrations of cells required for experiments.

2.2. Culture tests

At first, *E. durans* E204 was inoculated in MRS broth and skimmed milk. The inoculated samples were incubated at 30 ℃ for 48 h. Microbial counts, antimicrobial activity of cell free supernatant (CFS) against *L. monocytogenes* and pH values were determined each two hours.

For challenge tests, *E. durans* (10^8 CFU/ml) was co-cultured with *L. monocytogenes* (10^6 CFU/ml) in 100 ml of skimmed milk. In this test, two separate controls were considered; the first one was inoculated with *L. monocytogenes* alone, and the second one was inoculated with *E. durans* alone.

For testing the supernatant of *E. durans* culture against *L. monocytogenes* in milk, two groups of three mono-cultures of *L. monocytogenes* were prepared at 10^6 CFU/ml and 10^4 CFU/ml. Both groups were supplemented, at the onset of growth in milk, with 10, 50 and 100 ml of neutralized and filter-sterilized cell free supernatant (CFS) harvested after 18 hours of *E. durans* growth in skim milk. Two controls (10^6 CFU/ml and 10^4 CFU/ml) of *L. monocytogenes* without CFS were carried out in skim milk.

The cultures were incubated at 30°C and regularly sampled for microbiological and physicochemical analyses. Values of pH were determined using a pH meter 210 (HANNA instruments, Romania). Mean values of two measurements were recorded for each sample.

2.3. Microbial enumeration

Microbial analysis was performed at the indicated intervals of time. Each 0.1 ml sampled was 10-fold serially diluted in physiological saline solution. Then from appropriate dilutions, 0.1 ml was spread-plated in duplicate. *Enterococcus durans* cell number was determined on Slanetz and Bartly agar (Biokar Diagnostics, Beauvais, France). *Listeria monocytogenes* was enumerated in Palcam Agar (BK145, Biokar Diagnostics, Beauvais, France) with selective supplement (BS00408, Biokar Diagnostics, Beauvais, France). Plates were incubated at 30°C for 48 hours and colonies counted manually.

2.4. Antibacterial activity

At the indicated intervals of time, samples from culture of *E. durans* were centrifuged at 8000g for 20 min at 4°C (Hettich Zebtrifugen, Universal 320, Andreas Hettich GmbH & Co. KG). Cell free supernatant was adjusted to pH 6, filter-sterilized and stored at - 20°C until used. Antibacterial activity in the CFS was evaluated against *L. monocytogenes* using the well diffusion assay. Mueller Hinton Agar (MHA) (Biokar Diagnostics) was first dispensed in sterile Petri dishes and, after solidification, overlaid with 6 ml of BHI soft agar (45°C) inoculated with *L. monocytogenes* (100 µl of overnight culture at ≈ 10^7 CFU/ml). Wells were filled with 100 µl of two-fold serial dilutions of CFS and the plates were incubated overnight at 37°C. The titre of bacteriocin-like inhibitory substance (BLIS) present in CFS was expressed as an arbitrary unit per millilitre (AU/ml), corresponding to the reciprocal of the highest dilution, causing obvious inhibition zone on the indicator strain, multiplied by a factor of 10.

2.5. Mode of action of the bacteriocin-like inhibitory substance

20 ml of CFS, previously filter-sterilized and neutralized, were added to 100 ml of a log-phase of *L. monocytogenes* CECT 4032 (4 hours) in BHI broth. Optical density readings at 620 nm of target strain were determined during 48 hours. *Listeria monocytogenes* culture without added CFS was used as control.

3. Results

3.1. Inhibitory activity in MRS broth and skimmed milk

In MRS broth, *E. durans* increased gradually, reached a maximum population level of 12.4 log CFU/ml after 10 hours and remained stable up to 48 hours of incubation. The antibacterial activity against *L. monocytogenes* was detected after 4 hours of incubation. The antibacterial activity started at the beginning of the log phase of *E. durans* growth, and increased gradually to reach a maximal level of 320 AU/ml after 10 hours. This level of activity remain constant a long the stationary phase (Figure 1a). Meanwhile, pH decreased after 2 hours of incubation to reach 4.1 after 48 hours.

In skimmed milk, *E. durans* reached a count of 12.4 log CFU/ml after 12 hours and remained stable up to 48 hours of incubation. The antibacterial activity (10 AU/ml) against *L. monocytogenes* was observed after 4 hours of incubation, reaching a maximum of 320 AU/ml after 12 hours and remained stable a long the stationary phase (Figure 1b). The pH of the medium decreased from 6.18 to 4.2 during bacterial growth.

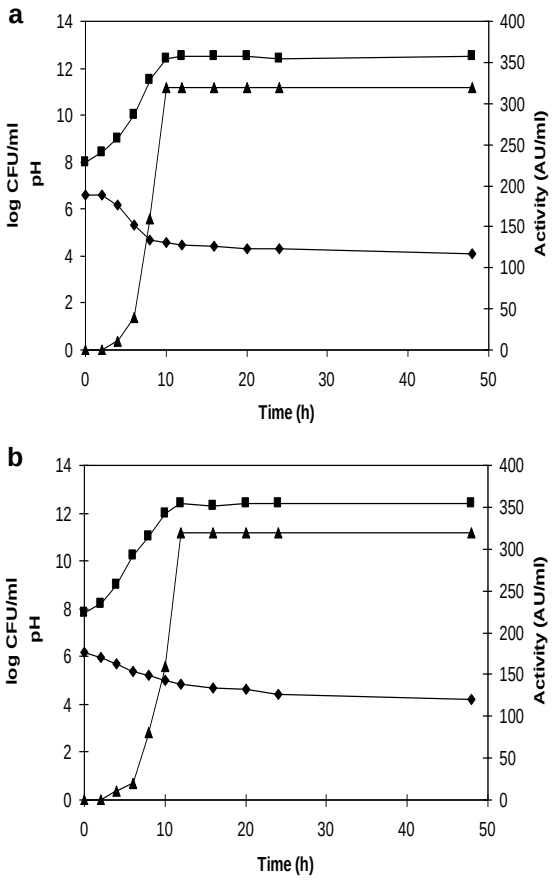

Figure 1. Growth of *E. durans* E204 in MRS broth (**a**) and skimmed milk (**b**). Growth (■), antibacterial activity against *L. monocytogenes* (▲) and pH (♦).

3.2. *Listeria monocytogenes* inhibition in the presence of *E. durans*

In skimmed milk, the growth of *L. monocytogenes* in the absence of *E. durans*, increased rapidly and reached approximately 11.8 log CFU/ml after 12 hours of incubation, which correspond to 5.8 log increase from the initial inoculum (Figure 2). However, when co-cultured with *E. durans* (initially at 8 log CFU/ml), *L. monocytogenes* (initially at 6 log CFU/ml) was suppressed after 16 hours of incubation (Figure 2), while the growth of *E. durans* continued to occur normally which showed the antagonist effect towards *L. monocytogenes*. The pH values of the co-cultivated samples (Figure 2) were similar to the pH values of the *E. durans* mono-culture samples (Figure 1b) over the entire incubation period.

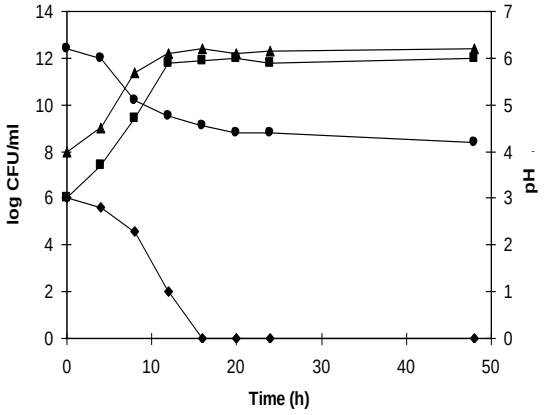

Figure 2. Growth evolution of co-cultured *L. monocytogenes* with *E. durans* E204 in skimmed milk during 48 hours at 30 ℃. *E. durans* counts (▲) in co-cultivation. *L. monocytogenes* counts in culture control (■) and in co-cultivation with *E. durans* (♦). pH of the medium in co-cultivation (●).

3.3. Survival of *L. monocytogenes* in the presence of cell free supernatant

The survival study of *L. monocytogenes* was performed in skimmed milk supplemented with three different volumes (10, 50 and 100 ml) of cell free supernatant harvested from 18 hours of *E. durans* cultured in skimmed milk (Figure 3). A rapid decrease in cell number occurred at the first four hours of incubation for all CFS aliquots, but a slight regrowth of the pathogen was observed thereafter. The reduction in cell number was positively correlated with CFS volumes. At 100 ml of CFS, *L. monocytogenes* recorded approximately 3 log CFU/ml lower than the initial cell number.

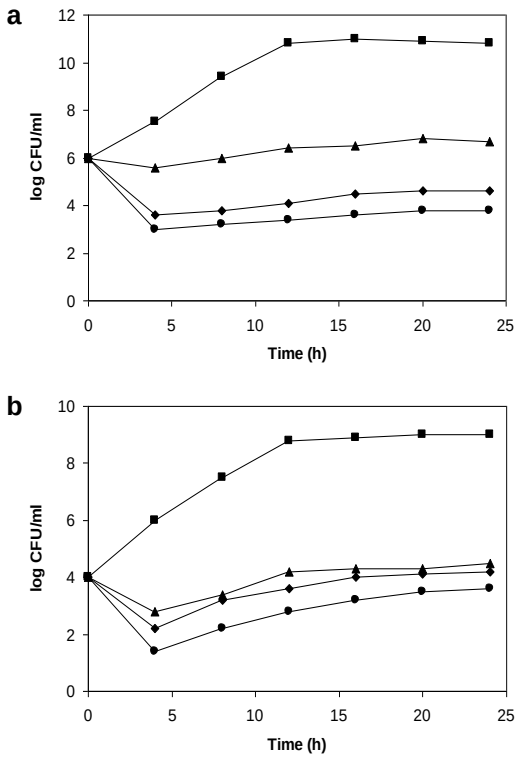

Figure 3: Survival of *L. monocytogenes* in skimmed milk supplemented with CFS obtained from 18 hours of *E. durans* culture. Different volumes of CFS: (■) 0 ml, (▲) 10 ml, (♦) 50 ml and (●) 100 ml applied to the pathogen strain at two different concentrations (**a**) 10^6 CFU/ml and (**b**) 10^4 CFU/ml.

3.4. Mode of action of the bacteriocin-like inhibitory substance

The addition of CFS at 4 hours of incubation of *L. monocytogenes* resulted in a sharp decrease of 0.6 in optical density (OD) at 620 nm compared to the control (Figure 4). Thereafter, the difference in OD remained unchanged.

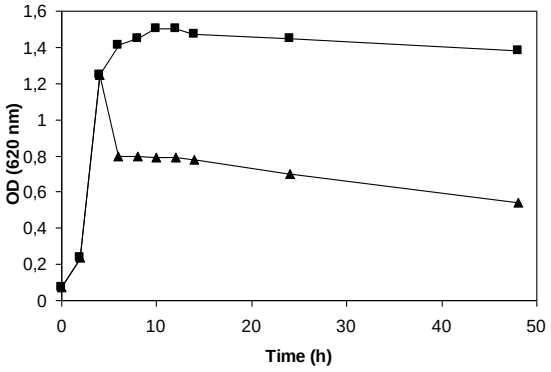

Figure 4. CFS effect on the growth of *L. monocytogenes*. Optical Density (OD_{620nm}) of treated culture (▲) and control culture (■).

4. Discussion

Enterococcus durans E204 used in the present study is a candidate strain for a safe practical use. In a previous study (Khay et al.2013), the results of the pathogenicity tests performed showed that *E. durans* was not able to decarboxylate lysine, histidine, ornithine, or tyrosine and was susceptible to vancomycin, kanamycin, tetracyclin, ampicillin, rifampicin and chloramphenicol.

Several studies have demonstrated the antagonistic activity of lactic acid bacteria isolated from dairy products against *L. monocytogenes* (Nero et al.2009; Benkerroum et al.2000). Bacteriocins from LAB strains are indicated to prevent the growth of undesirable and pathogenic bacteria in food. In this study, the antimicrobial effect of bacteriocin-like inhibitory substance produced by *E. durans* E204 on *L. monocytogenes* was investigated in skimmed milk.

We first, assessed the production of BLIS by *E. durans* in MRS broth and skimmed milk (Figure 1). It should be noted that bacterial growth behaved similarly in both skimmed milk and MRS broth. This was mainly the case of *E. durans* E204, isolated from camel milk, whose population as well as BLIS titre reached a maximum of 12.4 log CFU/ml and 320 AU/ml respectively after 12 hours of incubation in both media. This study showed that BLIS production is dependent on the biomass concentration. These results confirmed the previous studies which showed that the biosynthesis of bacteriocin was maximal at the end of the exponential phase (Yamazaki et al.2003; Campos et al. 2006).

On the other hand, the co-culture test carried out in skimmed milk using both *L. monocytogenes* and *E. durans* revealed for the first time the inhibitory effect of *E durans* on the pathogenic food-borne *L. monocytogenes* (Figure 2). The inhibition did not seem to be correlated with the reduction in pH during the first 16 hours of fermentation, confirming that the antimicrobial activity of *E. durans* is not due to the production of organic acid but it is clearly linked to the production of bacteriocin-like inhibitory substances. In co-culture with *E. durans*, no *L. monocytogenes* growth was detected and cells did not survive beyond 16 h. This could be due to the production of a sufficient amount of BLIS inactivating the respective growing cells of *L. monocytogenes*.

Different cell free supernatant aliquots were assessed and the inhibition of *L. monocytogenes* growth was observed as well as cell number decreased very fast (Figure 3). Prevention of *L. monocytogenes* increased with increasing CFS volume. These results showed that growth and survival of *Listeria ś* cells were strongly affected by

CFS which initially was very effective in reducing cell number. Such reduction was mostly due to the presence of BLIS in CFS. The initial decline in cell numbers was followed by regrowth of the target strain, indicating that the amount of BLIS concentration was insufficient to inhibit all the cells of the target microorganism.

It is well known that the mode of inhibition of bacteriocins depends on the concentration and on the nature and the physiological stage of the target strain. The BLIS produced by *E. durans* showed a rapid bactericidal effect towards *L. monocytogenes* since it caused depletion in optical density of the indicator strain. The decrease in OD_{620nm} indicated cell lysis (Figure 4). This was similar to the bactericidal mode of action with concomitant lytic effect of enterocin L50A and L50B (Cintas et al.1998) and enterocin 012 (Jennes et al. 2000). In general, enterocins display a bactericidal effect towards sensitive strains (Gálvez et al.1998). To state the bactericidal or bacteriostatic mode of action of bacteriocins, several factors need to be considered such as assay systems used, concentrations and purity of the inhibitor, the sensitivity of the indicator species, the density of the cell suspension used and the type of buffer or broth used (Jeevaratnam et al.2005).

In conclusion, it is important to mention that the challenge level of *Listeria* used in the present study was very high (ca. 10^6 CFU/ml) considering the actual level of *Listeria* encountered in milk which is in most cases less than 10^3 cells/ml (Beckers et al. 1987; Meyer-Broseta et al.2002). This study has shown that *E. durans* E204 inoculated in skimmed milk, exhibits interesting properties that make it a strong candidate for practical use as biopreservative culture to decrease the risk of listeriosis.

Acknowledgements

The authors would like to thank Dr. Bouchra Edderkaoui Research Scientist from Loma Linda Veterans Association for Research and Education, Loma Linda University for valuable comments and the corrections of the manuscript.

References

[1] Benkerroum N, Oubel H, Zahar M, Dlia S, Filali-Maltouf A (2000). Isolation of a bacteriocin-producing *Lactococcus lactis subsp. lactis* and application to control *Listeria monocytogenes* in Moroccan jben. J Appl Microbiol. 89(6): 960-968.

[2] Beckers HJ, Soentoro PSS, Asch EHMD-v (1987). The occurrence of *Listeria monocytogenes* in soft cheeses and raw milk and its resistance to heat. Int J Food Microbiol. 4: 249-256.

[3] Campos CA, Rodríguez O, Calo-Mata P, Prado M, Barros-Velásquez J (2006). Preliminary characterization of bacteriocins from *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (Psetta maxima). Food Res Int. 39(3): 356-364.

[4] Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Håvarstein LS (1998). Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J Bacteriol. 180(8): 1988-1994.

[5] Coventry MJ, Gordon JB, Wilcock A, Harmark K, Davidson BE, Hickey MW, Hillier AJ, Wan J (1997). Detection of bacteriocins of lactic acid bacteria isolated from foods and comparison with pediocin and nisin. J Appl Microbiol. 83(2): 248-258.

[6] Davies EA, Bevis HE, Delves-Broughton J (1997). The use of the bacteriocin, nisin, as a biopreservative in ricotta-type cheeses to control the food-borne pathogen *Listeria monocytogenes*. Lett Appl Microbiol. 24(5): 343-346.

[7] Farber JM, Peterkin PI (1991). *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev. 55(3): 476-511.

[8] Fenlon DR (1986). Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. Vet Rec. 118(9): 240-242.

[9] Gálvez A, Valdivia E, Abriouel H, Camafeita E, Mendez E, Martínez-Bueno M, Maqueda M (1998). Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Arch Microbiol. 171(1): 59-65.

[10] Griffith M, Deibel KE (1989). Survival of *Listeria monocytoenes* in yogurt with varying levels of fat and solids. J Food Safety. 10 (3): 219-230.

[11] Jeevaratnam K, Jamuna M, Bawa AS (2005). Biological preservation of foods- bacteriocins of lactic acid bacteria. Indian J Biotechnol. 4: 446-454.

[12] Jennes W, Dicks LM, Verwoerd DJ (2000). Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. J Appl Microbiol. 88: 349-357.

[13] Khay EO, Idaomar M, El Moussaoui N, Abrini J (2013). Application of a bacteriocin-like inhibitory substance producing *Enterococcus durans* E204 strain, isolated

from camel milk, to control *Listeria monocytogenes* CECT 4032 in goat *jben*. Ann Microbiol. DOI: 10.1007/s13213-013-0666-1.

[14] Khay EO, Idaomar M, Pastrana Castro LM, Fajardo Bernárdez P, Skali Senhaji N, Abrini J (2011). Antimicrobial activities of the bacteriocin-like substances produced by lactic acid bacteria isolated from Moroccan dromedary milk. Afr J Biotechnol. 10(51): 10447-10455.

[15] Khay EO, Pastrana Castro LM, Fajardo Bernárdez P, Skali Senhaji N, Idaomar M, Abrini J (2012). Growth of *Enterococcus durans* E204 producing bacteriocin-like substance in MRS Broth: description of the growth and quantification of the bacteriocin-like substance. Afr J Biotechnol. 11(3): 659-665.

[16] Mahmoodi MM (2010). Occurrence of *Listeria monocytogenes* in raw milk and dairy products in noorabad, Iran. J Anim Vet Adv. 9(1): 16-19.

[17] McAuliffe O, Hill C, Ross RP (1999). Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture. J Appl Microbiol. 86(2): 251-256.

[18] Meyer-Broseta S, Diot A, Bastian S, Riviere J, Cerf O (2002). Estimation of low bacterial concentration: *Listeria monocytogenes* in raw milk. Int J Food Microbiol. 80: 1-15.

[19] Muganpoza D, Muyanja CMBK, Ogwok P, Serunjogi ML, Nasinyama GW (2011). Occurrence of *Listeria monocytogenes* in bulked raw milk and traditionally fermented dairy products in Uganda. Afr J Food Agricul Nutr Dev. 11(2): 4610-4622.

[20] Nero LA, Mattos MR, Beloti V, Barros MAF, Ortolani MBT, Franco BDGM (2009). Autochthonous microbiota of raw milk with antagonistic activity against *Listeria monocytogenes* and *salmonella enteritidis*. J Food Safety. 29(2): 261-270.

[21] Ortolani MBT, Moraes PM, Perin LM, Viçosa GN, Carvalho KG, Silva Júnior A, Nero LA (2010). Molecular identification of naturally occurring bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft cheese. J Dairy Sci. 93(7): 2880-2886.

[22] Pan Y, Breidt JF, Kathariou S (2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol. 72(12): 7711-7717.

[23] Piccinin DM, Shelef LA (1995). Survival of *Listeria monocytogenes* in cottage cheese. J Food Prot. 58(2): 128-131.

[24] Rocourt J (1996). Risks factors for listeriosis. Food Control. 7(4): 195-202.

[25] Rodríguez E, Tomillo J, Nuñez M, Medina M (1997). Combined effect of bacteriocin-producing lactic acid bacteria and lactoperoxidase system activation on *Listeria monocytogenes* in refrigerated raw milk. J Appl Microbiol. 83(3): 389-395.

[26] Schillinger U, Geisen R, Holzapfel WH (1996). Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci Technol. 7(5): 158-164.

[27] Vogel BF, Jørgensen LV, Ojeniyi B, Huss HH, Gram L (2001). Diversity of *Listeria monocytogenes* isolates from cold-smoked salmon produced in different smokehouses as assessed by Random Amplified Polymorphic DNA analyses. Int J Food Microbiol. 65(1-2): 83-92.

[28] Wan J, Harmark K, Davidson BE, Hillier AJ, Gordon JB, Wilcock A, Hickey MW, Coventry MJ (1997). Inhibition of *Listeria monocytogenes* by piscicolin 126 in milk and Camembert cheese manufactured with a thermophilic starter. J Appl Microbiol. 82(3): 273-280.

[29] Yamazaki K, Suzuki M, Kawai Y, Inoue N Montville TJ (2003). Inhibition of *Listeria monocytogenes* in cold-smoked salmon by *Carnobacterium piscicola* CS526 isolated from frozen surimi. J Food Prot. 66(8): 1420-1425.

6/20/2013