Study the Effect of Lactobacillus on the Prevalence of Some Aerobic and Anaerobic Microorganisms in Dry Sausage

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Abstract: Fermented foods attributed in improvement of nutritional value and safety against bacterial pathogens. *Lactobacillus plantarum* sp. (TN 635) was used in fermentation of meat sausage, using meat from the local market in Giza and sterilized by radiation at 10 kGy using the cobalt 60 Egypt Gamma-1 irradiator to obtain complete sterility avoiding any inferring factors during experiment. The prepared sausages were divided into two groups the first was inoculated with *Staph. aureus*, *E.coli* and *Cl Perfringens* and the second was inoculated with *L .plantarum* with the three pathogenic bacteria under investigation *Staph aureus, E.coli,* and *Cl.perfringens*. The assessment of the microbial growth indicated that the effect of *L.plantarum* decreased *E.coli* growth on the third day and decreased *Staph.aureus* on the third day while with *Cl.perfringens* the decrease was on day 10. The *L.plantarum* showed marked effect on the *Staph.aureus* and *E.coli* growth towards decreasing as it was increasing itself in count. It was observed that it's significant ($p< 0.01$) higher in count and significant ($p>0.01$) lower in pH, odor and flavor.
[Amal A. Shehata, Shireen M. Nosier, Kawther A. Ismail and Waffaa S. Mohamed. **Study the Effect of Lactobacillus on the Prevalence of Some Aerobic and Anaerobic Microorganisms in Dry Sausage**. *N Y Sci J* 2013;6(3):58-64]. (ISSN: 1554-0200). http://www.sciencepub.net/newyork. 9

Key wards: Dry sausage, L.plantarum, E.coli, Cl.perfringens and Staph.aureus

1. Introduction:

Bacteriocins are biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species. Numerous strains of bacteriocin producing bacteria have been isolated in the last two decades from different ecological niches including meat, fish, and milk (**Svtoslav**, **2009**).

Lacobacillus plantarum sp. Strain, its antimicrobial compound was determined as a protinaceous substance, stable to heat and to treatment with surfactants and organic solvents. Highest antimicrobial activity was found between pH 3 and 11 with an optimum at pH- 7. The maximum bacteriocin production (5,000 Au/ml) was recorded after a 16-hrs incubation in Man, Rogosa, and Sharpe (MRS) medium at 30°C (**Slim *et al.*, 2010**).

Food security, the availability of food and its accessibility to people, has been an important concern in most developing countries where food preservation techniques have been very inadequate (**Postnote**, **2006**).

Some of the lactic acid bacteria associated with starter cultures in fermented meat products are *pediococcus acidilactici*, *p.pentosaceus*, *Lactobacillus plantarum*, *Lb. sake* and *Lb. curvantus* among others (**Rodriguez *et al.*, 1995**; **Aymerich, 1996**).

Traditional sucuks are well-known and very popular meat products. Nawadays, sucuks are produced at butcher shops and by manufacturing companies from sheep and/or beef meat, beef fat or tail fat, salt, sugar, nitrite/nitrate, garlic and various spices such as black pepper, red pepper, cumin, cinnamon, allspice, and clove (**Bozkurt** and **Erkmen, 2003; Aksu** and **Kaya, 2004**).

Like all fermented sausages, sucuks have a long storage life due to the added salt, the antimicrobial compounds such as additive (nitrite, nitrate), low pH and water activity (**Bozkurt** and **Erkmen, 2003**; **Soyer *et al.*, 2005**).

The quality of the products present on the market is variable. The manufacture of good products with a standard quality is possible only if the meat are the dominant useful homofermentative strains of lactic acid bacteria are present (**Coretti**, **1971**, **1975**).

Fermented foods have many advantages attributes such as improved nutritional value and safety against bacterial pathogens (**Gadaga *et al.*, 2004**).

Lacobacillus plantarum isolated by **Enan *et al.*** (**1996**) from dry sausage produced an antimicrobial substance that inhibited other strains of the genera *Lactobacillus* and *Lactococcus*, and some foodborne pathogens including *Listeria monocytogenes* and *clostridium perfringens*. Also has bactericidal effect against *Staphylococcus aureus* (**Muller *et al.*, 2009**). *Lactobacillus plantarum* inhibited the growth of pathogenic microorganisms such as *E.coli* and *Staph. Aureus* (**Nedelcheva *et al.*, 2010**).

As one possible mode of action for probiotics is the production of antimicrobial compounds, Lactic acid bacteria may act as both probiotic and bioprotective culture as well as fermenting agent in

meat product, such as sausage (**Työppönen *et al.*, 2003**).

Lactobacilli are the predominant lactic acid bacteria and among them the most frequently isolated strains are *Lactobacillus curvatus*, *Lactobacillus sakei*, and *Lactobacillus plantarum* (**Schillinger** and **Liicke, 1987** and **Hammes, 1990**).

The most promising bacteria for starter cultures are those which are isolated from the indigenous microflora of traditional products. These microorganisms are well adapted in the meat environment and are capable of dominating the microflora of products. The strains selected as starter or protective cultures must have the most important technological properties and/or bacteriocin production capabilities (**Hammes**, **1990**).

2. Material and Methods

Organoleptic Examination:

The fermented sausage samples were sensory evaluated by a panel group of 10 members, randomly selected from the staff members, researchers and PhD students of the Food Hygiene Department, Animal Health Research Institute, Cairo, Egypt according to **Hemat *et al*., 2009**.

pH measurement:

The pH was determined during the fermentation and ripening of dry fermented sausage by blending sausage samples with distilled water (1:10) and the pH values of the suspensions were determined by pH meter directly as the mean value of three measurements after 3,7, and 15 days of fermentation and ripening (**Hemat *et al*., 2009**).

Culture media:

The culture media used are peptone water, Mann Rogosa and Sharpe (MRS) agar and broth (Oxoid), Eusien Methylene blue (EMB) agar (Lab M), *Cl.perfringens* agar (Biolif), D.cyclocerine supplement (Biolif), Baird parker agar (Himedia), Tellurated egg yolk emulsion (Himedia).

Microbiological cultures:

Lactobacillus plantarum sp. (TN 635) was obtained from Ain-Shams University. *Lactobacillus plantarum* was reactivated by three consecutive subculturing on (MRS) broth then incubated anaerobically at 37ºC for 24hrs, (**Hull** and **Robert, 1984**). Then it is adjusted to obtain the desired inoculums level 10^8 cfu/mL.

The pathogens used are *Staphylococcus aureus* (7447/6538p), *Escherichia coli* (12923/8739) and *Cl.perfringens* (8237/13124) was obtained from the reference strain bank of food hygiene department Animal Health Research Institute (AHRI).

Inoculum preparation: Each strain was deep frozen stored in a cryo protective vial containing preservative solution at -70ºC.

Cryo bead (inoculum) of each strain was cultivated in Tryptic Soy Broth overnight at 35ºC. Then cells were centrifuged for 10 min at 8000 rpm. Supernatant was discarded and cells were washed three times and re-suspended in sterile 0.1% peptone water. The cells were diluted in peptone water adjusted to obtain the desired inoculum level (10^4cfu/ml), (**Osman, 2008**).

Preparation of fermented sausage and inoculation:

A total of 3.5 Kg of imported deep frozen beef chuck was purchased from the local Market in Giza governorate. Spices were prepared to make fermented sausage dough according to **Osman (2008**).

Radiation process for sterilization: Minced meat, spices and beef natural casing were packed in polyethylene package and sterilized by irradiation at 10 kGy according to **Hammad *et al*. (2003**). The irradiation process was carried out using the cobalt 60 Egypt Gamma-1 irradiator, located at the National Centre for Radiation Research and Technology (NCRRT) Nasr City, Cairo, Egypt. Sausage were prepared by adding 2.5% sodium chloride, 100ppm sodium nitrite and spices were added and mixed with minced meat, then divided into two groups. The control group (A) was divided into four parts (each 500 gm). The first part was inoculated with *Lactobacillus plantarum* to a concentrate sufficient to provide $1x10^8$ cfu/gm (**FADIA NAIM *et al*., 2003**). The second part was inoculated with *Staph aureus* to reach final concentration in sausage $1x10^4$ cfu/gm, the third was inoculated with $1x10^4$ cfu/gm *E.coli* and the fourth with $1x10^4$ cfu/gm *Cl.perfringens.*

The second group (B) inoculated with *Lactobacillus plantarum* in conc. of 10^8 cfu/gm then divided into 3 equal parts (each 500 gm): the 3 parts was inoculated with a final bacterial concentration of 10^4 cfu/gm of *Staph aureus, E.coli* and *Cl.perfringens*, respectively. The meat was mixed in stomacher bags using stomacher. Then sausage daugh were stuffed into cattle natural casing (the 7 parts separately). Then ripened and refrigerated (**Osman 2008**).

▪**Assessment of microbial growth**:

Analysis was conducted on sausage dough at zero time/days 1, 2,3,4,5,7,10 and 15 of production. Counting the bacterial load was applied according to **A.P.H.A., 2001** and **FDA, 2001** for (*Cl.perfringens*).

Twenty five gm of each sample was taken on 225 ml of peptone water and homogenized in a stomacher in polyethylene bag.

The samples were serially diluted under aseptic condition. A portion 0.1 ml of each dilution was aseptically inoculated in its appropriate media plates and spreeded (for *Cl.perfringens* 1 ml of diluted

sample then poured another layer of media and left to solidify).

Media used for *E.coli* was Eusien Methylene blue (EMB) at 35°C/24hrs incubation.

Media used for *Staph. Aureus* was Baird parker agar (B.P.) at 35°C/24hrs incubation.

Media used for *Cl. Perfringens* was *Cl. Perfringens* Agar (Tsc) at 35°C/24hrs incubation.

Media used for *L.plantarum* was Mann Rogosa and Sharsp agar (MRS) under anaerobic conditions using Gas pack anaerobically for at 37°C/24hr according to **Pornpan *et al.* (2010)**. The total counts of colonies were recorded at each interval to monitor their growth.

Statistical analysis:

For statistical analysis, average counts of colonies on duplicate plates were transformed into log $CFUg^{-1}$.

3. Results and Discussion

The experimental sausage samples were analyzed to determine the presence of the inoculated *Staph. Aureus, E.coli* and *Cl.perfrringens* and study the effect of *L.plantarum* on their growth. Using the descriptive attributes the sensory profiles of fermented sausage samples with *L.plantarum* at the end of ripening period Figs. 1, 2, 3 and 4 were produced. It was observed that it's significant ($P< 0.01$) higher in count and significant ($P>0.01$) lower in pH, odor and flavor.

The growth patterns of *E.coli, Staph aureus, Cl.perfringens* inoculated with *Lactobacillus plantarum* in fermented sausage and without *Lactobacillus plantarum* is presented in figs. (2), (3) and (4).

In fig (2) *E.coli* inoculated without *L.plantarum* increased gradually till reaching maximum beak of growth in day 4 ($1.6x10^6$) then gradual decrease reaching (3x10) in day 15.

At 7 days of fermentation the pH values of the sausages were 5.1, 5.0 and 4.9. *E.coli* inoculated with *L.plantarum* reached its beak of increasing in the second day ($7.2x10^5$). Then gradual decrease occurred starting from day 3 ($2.2x10^5$) gradually then sharp decrease in day 7 ($1x10^2$). Finally at the end of ripening time (15 day) were < 10 and the pH of the sausages fermented by *L.plantarum* were 4.9 while the pH of the control was 5.0.

In fig (3) *Staph aureus* inoculated without *L.plantarum* increased in the 2nd and 3rd day till reaching ($3.3x10^5$) then gradual decrease reaching $1x10^2$ cfu/gm in day 7. While *Staph aureus* inoculated with *L.plantarum* increased in the 2nd day then decreased gradually from day 3 ($8x10^3$) to be sharp in day 7 reaching 1x10 in day 15.

In fig (4) *Cl.perfringens* inoculated without *L.plantarum* increased gradually till day 10 ($1.9x10^7$) then slightly decreased in day 15 ($7.7x10^6$). While increasing of *Cl.perfringens* occurred in the samples having *L.plantarum* till day 7 ($1.68x10^6$) then started to decrease in day 10 with sharp decrease in day 15 was ($1x10^2$).

Fig (5) indicated that pH values of the fresh sausage were significantly ($p<0.01$) higher than those during the ripening period at 25°C. The pH values of samples made from *L.plantarum* were significantly ($p<0.01$) lower than control sample from the beginning and during ripening period. The data revealed that, there was a continuous decrease in pH values in all samples during 15 days of ripening period of fermented sausage reflecting the production of the bacterial metabolites, then exhibited a slight rise in pH values after storage at 25°C. These results coincided with those of **Montet *et al* (2009)**.

It was suggested that, the pH values increase in the later stages of the ripening period is related to the formation of peptides, amino acids and ammonia as a result of proteolysis as reported by **Montet *et al* (2009)**.

The effect of *L.plantarum* appeared to minimize growth of *E.coli* in 3rd day of inoculation with *L.plantarum* count reaching ($2.5x10^9$) and its effect on *Staph. aureus* growth appeared in day 3 with *L.plantarum* count ($2.5x10^9$) while the effect on *Cl.perfringens* appeared clear on day 10 and increased on day 15 with count of *L.plantarum* was $4.5x10^{11}$ and $2x10^{12}$ for *L.plantarum* as shown in figs. 2, 3 and 4.

The effect of *L.plantarum* reducing *E.coli* and *Staph aureus* growth appeared on day 3 and *E.coli* disappeared on day 15 while *Staph. Aureus* was 1x10 on day 15. While the obvious decrease of *Cl.perfringens* growth on day 10 needed high *L.plantarum* count.

Similar investigation;

E.coli was inhibited by *L.plantarum* as it was increased within the first 36h then decreased and completely extincted after 96h of fermentation (**Obadina *et al.* 2006**) also *L.plantarum* inhibited ($P< 0.001$) growth of *E.coli* due to effect of lactic acid produced (**Murry, 2004**) while *L.plantarum* inhibited growth of *E.coli* at temp 15-18° C in the meat products (**Nedelcheva *et al.*, 2010**).

Other similar investigations showed the effect of *L.plantarum* on *Staph aureus* growth after 24h by 1.6 Log and disappeared after 72h (**Mami *et al.*, 2008**) also *Staph aureus* during fermentation process of Nham its number was slightly increased in the 1st day and then decreased after ward (**Chokesajjawattee *et al.*, 2009**) and number of *Staph aureus* in fermented meat products was slightly increased in the 1st day then decreased (**Chokesażżawatee *et al.*, 2009**). Mostly *L.plantarum*

was shown to have a bactericidal effect against *Staph aureus* (**Muller *et al*., 2009**). For *Cl.perfringens L.plantarum* inhibited ($P < 0.001$) growth of *Cl.perfringens* due to the effect of lactic acid produced (**Murry, 2004**) and *L.plantarum* produces an antimicrobial substance inhibits food born pathogens as *Cl.perfringens* (**Enan *et al*., 1996**).

Finally products fermented with *L.plantarum* as sausages have low pH value are safe as most pathogens are unable to survive as *Staph aureus*, *E.coli*, as *L.plantarum* exhibited high degree of inhibition and antimicrobial activity on the pathogens.

Fig. (1): Count of *L.plantarum* in ripenning periods

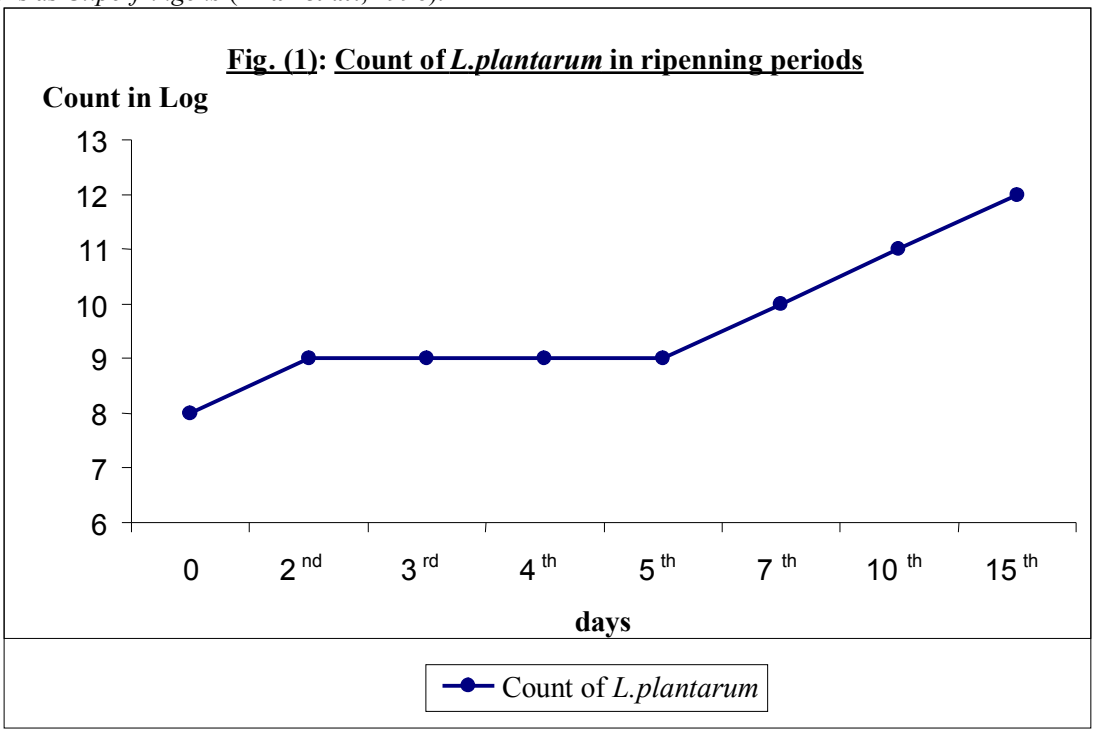
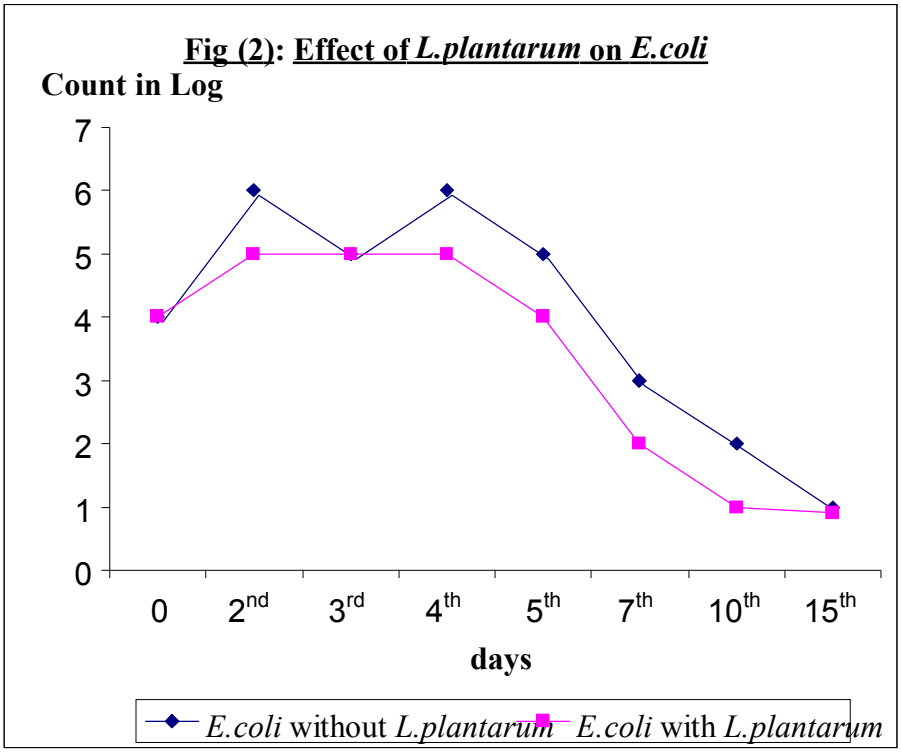


Fig (2): Effect of *L.plantarum* on *E.coli*



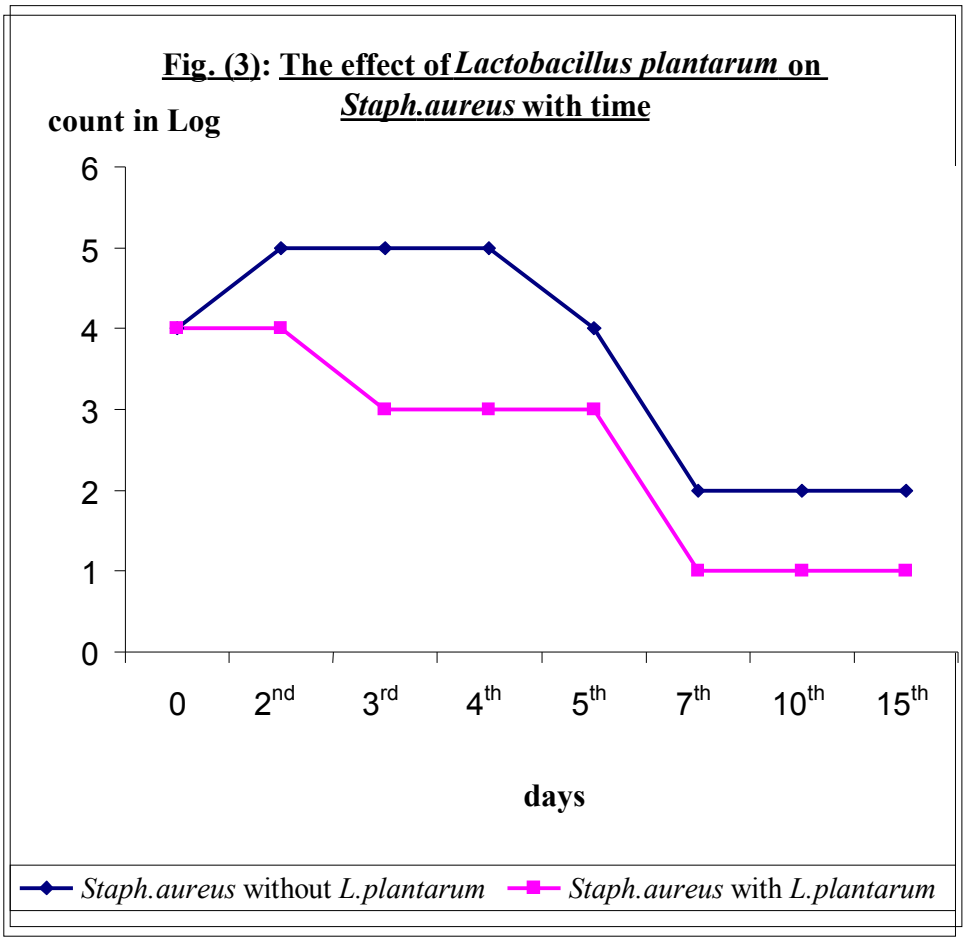


Fig. (3): The effect of Lactobacillus plantarum on Staph.aureus with time
count in Log
6
5
4
3
2
1
0
0
2nd
3rd
4th
5th
7th
10th
15th
days
Staph.aureus without L.plantarum
Staph.aureus with L.plantarum

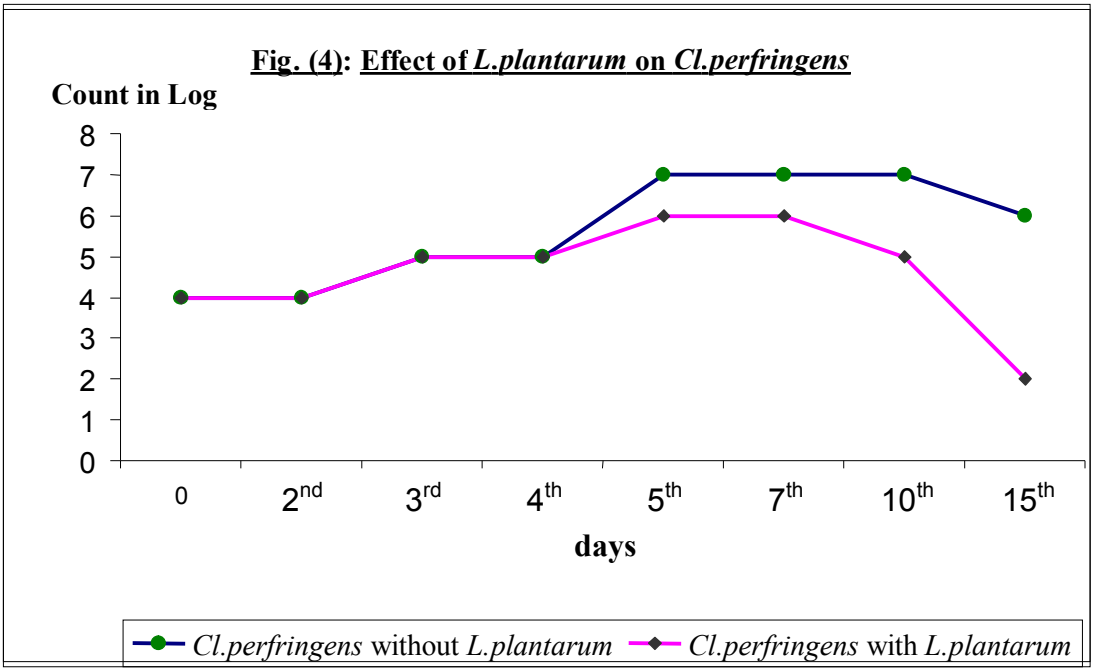
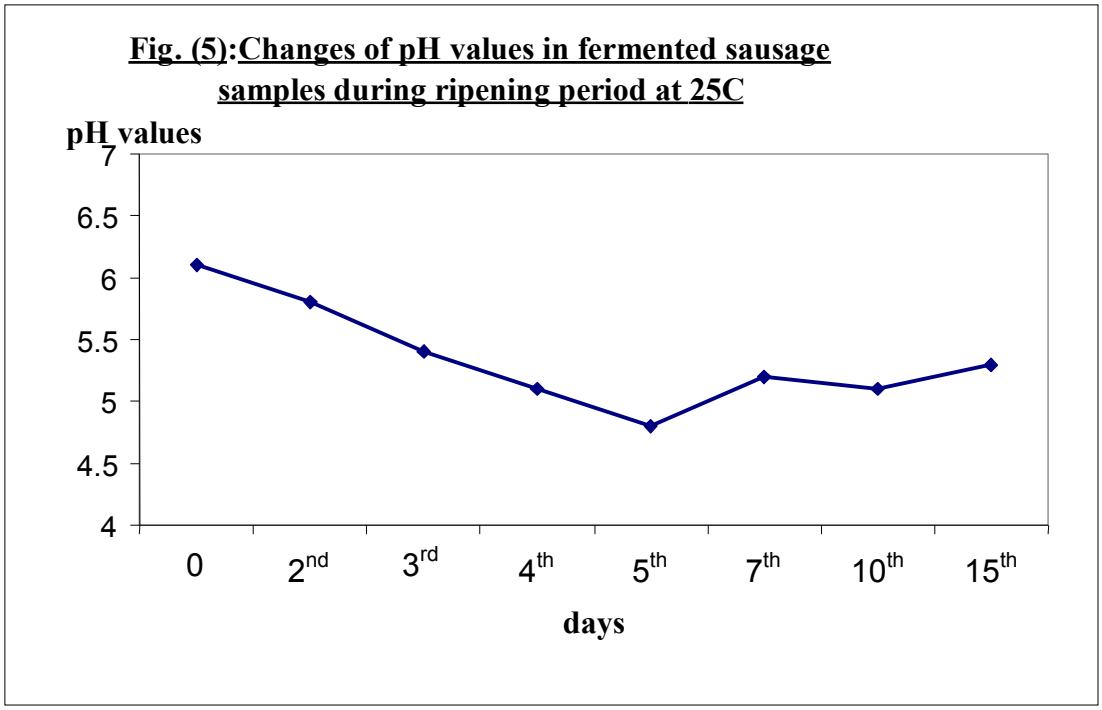


Fig. (4): Effect of L.plantarum on Cl.perfringens
Count in Log
8
7
6
5
4
3
2
1
0
0
2nd
3rd
4th
5th
7th
10th
15th
days
Cl.perfringens without L.plantarum
Cl.perfringens with L.plantarum



Recommondation:

Thus, it can recommend the use of *L.plantarum* to produce safe and highly nutritious dry fermented sausage with significant enhanced sensory characteristics.

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