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SURVIVAL OF *ESCHERICHIA COLI* O157:H7 CO-CULTURED WITH DIFFERENT LEVELS OF *PSEUDOMONAS FLUORESCENS* AND *LACTOBACILLUS PLANTARUM* ON FRESH BEEF

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ABSTRACT

The purpose of this study was to investigate the effect of different levels of *Pseudomonas fluorescens* (10^2 and 10^6 $\log_{10}$ cfu/ml) and *Lactobacillus plantarum (*10^2 and 10^4 $\log_{10}$ cfu/ml) on the growth of *Escherichia coli* O157:H7 on beef loins. Beef loins inoculated with *E. coli* O157:H7 and *P. fluorescens* were aerobically stored for 7 days at 4 ºC, while those inoculated with *E. coli* O157:H7 and *L. plantarum* were vacuum packaged and stored for 8 weeks at 4 ºC. Aerobic Plate Counts (APC), *E. coli* O157:H7 and either *P. fluorescens* or *L. plantarum* counts were determined at different storage intervals. For the aerobically packaged beef loins, *E. coli* O157:H7 was detected throughout the 7 day storage period regardless of the *P. fluorescens* level in the inoculum. For the vacuum packaged beef loins, similar inoculum levels of *E. coli* O157:H7 and *L. plantarum* allowed *E. coli* O157:H7 to survive until week 5 of storage, while a higher inoculum level of *L. plantarum* inhibited *E. coli* O157:H7 from week 3. Once fresh beef has been contaminated with *E. coli* O157:H7, the level of *P. fluorescens* in the background flora does not inhibit its survival and growth. However, under vacuum storage, the application of *L. plantarum* as a biopreservative inhibits the survival of *E. coli* O157:H7 on beef. The higher the level of *L. plantarum* in the system, the earlier the onset of the inhibition. Farmers and abattoirs have to strengthen preventive strategies to eliminate contamination of beef carcasses with *E. coli* O157:H7.

Key words: aerobic storage, vacuum package, *E. coli* O157:H7, *P. fluorescens*, *L. plantarum*

INTRODUCTION

There is evidence that some slaughter animals in South Africa shed *E. coli* O157:H7 at the time of slaughter, which creates an opportunity for this pathogen to be present on meat and meat products. The organism has been found in 19 % and 7 % of slaughter cattle feces and carcasses, respectively. Similarly, it was found in 8 % and 1 % of sheep feces and carcasses, and in 31 % and 1 % of horse feces and carcasses (Veterinary Services, Gauteng Provincial Department of Agriculture, unpublished). Spoilage and pathogenic bacteria compete for available substrates in food to survive. Psychrotrophic bacteria especially of the genus *Pseudomonas* are common on meat. These organisms have been identified in numerous studies as the major spoilage organisms in refrigerated fresh meats (2, 12) partly due to their ability to

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form quorum sensing facilitated biofilms (14).

In South Africa, the meat industry utilizes vacuum packing to extend the shelf life of beef products for transportation, which is at times over considerable distances, since the final destination of these products often includes neighboring countries (24). Lactic acid bacteria (LAB) are facultative anaerobes which are antagonistic against many microorganisms including spoilage and pathogenic bacteria (1). Previously, research focused on the inhibitory effect of *L. plantarum* on *E. coli* O157:H7 in a range of food products including acidic fruit juices (29), processed meat products (22), and ground beef (19, 25), but there is no evidence of work conducted on whole beef loins. Since Pseudomonads and LAB are natural contaminants of foods of animal origin, which can also be contaminated by *E. coli* O157:H7, it is important to understand how these will affect the survival and growth of *E coli*.O157:H7 on aerobically and vacuum stored beef. Furthermore, it was suggested that decontamination techniques used in developed countries led to very low numbers of background microorganisms on fresh meat, resulting in reduced competition between such microorganisms and food pathogens. Fresh food products that contain 10^5 cells/g of harmless microbiota are less likely to allow low numbers of pathogens to proliferate than ones that contain 10^3 cells/g (13). The aim of this study was to investigate the effect of different levels of competitive, spoilage bacteria, *P. fluorescens* and *L. plantarum* on the survival and growth of *E. coli* O157:H7 on aerobically and vacuum packaged stored (4 ºC) beef, respectively.

MATERIALS AND METHODS

Bacterial strains

E. coli O157:H7 strain UT 10, isolated from meat, was obtained from the Agricultural Research Council, Onderstepoort, South Africa. This strain was used for both experiments 1 and 2. For experiment 1 *P. fluorescens* ATCC 13525 strain was used (Kiwk stik; MediMark, France). For experiment 2 *L. plantarum* ATCC 8014 (MediMark) strain was used.

Preparation of beef loins

Vacuum packaged deboned beef loin (*M. longissimus dorsi*) was purchased from a local butchery. Under aseptic conditions, the external layer (approximately 1 cm) of the muscle was removed within a biological safety cabinet (Labaire, France). The internal portion was cut aseptically into 25 g blocks using a sterile metallic sampler. Two sterile pieces were subsequently analyzed to determine the total aerobic counts after sterilization, which were <10 $\log_{10}$ cfu/cm^2.

Preparation of working cultures

E. coli O157:H7 strain was cultivated in Brain Heart Infusion Broth (BHIB) CM 225 (Oxoid, Hamisphre, England) and incubated at 37 ºC for 24h. The *P. fluorescens* strain was grown on Pseudomonas Agar CM 559 with selective SR 103 and incubated at 25 °C for 72 hrs, while LAB for experiment 2 was cultivated on de Man, Rogosa and Sharpe, (MRS) agar CM 359, (Oxoid) and incubated at 30 °C for 48 hours. 0.5 McFarland standard (Andrew, 2005) was used to prepare cultures containing 10^2 $\log_{10}$ cfu/ml of *E. coli* O157:H7, as well as 10^2 and 10^6 $\log_{10}$ cfu/ml of both *P. fluorescens* and *L. plantarum.* Serial dilutions of the bacterial cultures were made using Buffered Peptone Water (BPW) CM 509, (Oxoid) and plated onto Plate Count Agar (PCA) CM 463, (Oxoid) for the determination of the exact number of cfu/ml. A cocktail inoculum of 300 ml was prepared by mixing 150 ml of each bacterial inoculum at the concentration of 10^2 $\log_{10}$ cfu/ml. Another cocktail inoculum was prepared by mixing 150 ml volumes of 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 inoculum with 10^6 $\log_{10}$ cfu/ml *P. fluorescens* and that of 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^4 $\log_{10}$ cfu/ml *L. plantarum*.

Inoculation of beef loins

For each treatment, sterile pieces of 25 g of beef were individually submerged into the inoculum for 10 min to allow

for bacterial attachment. Inoculated beef pieces were air-dried for 5 minutes. In experiment 1, the inoculated beef pieces were packaged individually in zip-lock plastic pouches (PVC, O_2 transmission>10000 cm^3/m^2 per 24h/atm) (150 mm × 180 mm× 40 mm) and stored aerobically at 4 °C for a total of 7 days. Duplicate samples were collected and analyzed after 0, 2, 3, 5 and 7 days to determine *E. coli* O157:H7and *P. fluorescens* counts, as well as the APC. The same procedure as in experiment 1 was used to inoculate sterile beef pieces, which were subsequently separately placed in vacuum bags (150 mm x 200 mm), vacuum sealed and stored at 4 ºC for 2 months. Samples were analyzed after 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks of storage for *E. coli* O157:H7, *L. plantarum* and APC. All experiments were carried out in triplicate with duplicate samples analyzed at each storage interval in duplicate plates.

Microbiological analysis of inoculated beef loins

During each sampling period, duplicate subsamples of 25 g were homogenized with 225 ml of 0.1 % BPW in a stomacher (Seward 400, Seward, London, United Kingdom) and subsequent decimal dilutions were prepared and plated. For the enumeration of *E. coli* O157:H7, sorbitol McConkey (SMAC) Agar CM 813, (Oxoid) with selective supplement SR 172, (Oxoid) was used. SMAC plates were incubated at 37 °C for 24 hours.

Statistical analysis

Data were analyzed by two way Analysis of variance (ANOVA) including the interaction effect using Statistica 7 (Statsoft Inc., Tulsa, Oklahoma, USA, 2003) to determine if levels of *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml), *P. fluorescens* (10^2 and 10^6 $\log_{10}$ cfu/ml) and *L. plantarum* (10^2 and 10^4 $\log_{10}$ cfu/ml) and storage time (Day 0, 2, 3, 5, 7) under aerobic storage and 8 weeks (1 week interval) under vacuum storage significantly (95% confidence interval) affect survival and growth of *E. coli* O157:H7 on meat. All samples were analyzed in duplicate and each experiment was repeated three times. Means were separated by Fisher's Least Significant Difference (LSD) analysis.

RESULTS

Effect of different levels of *E. coli* O157:H7 and *P. fluorescens* in the inoculum on the growth of *E. coli* O157:H7 on beef loins

The *P. fluorescens* inoculum level did not influence the survival and growth of *E. coli* O157:H7 ($p=0.62$), while growth of *E. coli* O157:H7 was affected by the storage time ($p<0.05$). There was a significant ($p<0.05$) interaction between the inoculation level and time (level x time) on the growth and survival of *E. coli* O157:H7. Inoculum level and storage time did not influence *P. fluorescens* count ($p=0.20$) and APC ($p=0.94$).

When similar levels (10^2 $\log_{10}$ cfu/ml) of *E. coli* O157:H7 and *P. fluorescens* were combined, *E. coli* O157:H7 was not recovered from beef (Fig 1a) on day 0. By day 2 of storage, the *E. coli* O157:H7 count reflected the level that was inoculated, 2.54 $\log_{10}$ cfu/cm^2. *E. coli* O157:H7 count significantly ($p<0.05$) increased until day 3. From then on, no further growth occurred. By day 7, the *E. coli* O157:H7 population was recoverable at levels of 4.44 $\log_{10}$ cfu/cm^2. *E. coli* O157:H7 increased by 2 log when comparing levels at days 2 and 7.

Under the same experimental conditions, *P. fluorescens* showed a 2 day lag phase, as the counts enumerated during day 0 and day 2 remained at the same level (Figure 1a). There was a significant ($p<0.05$) increase in growth by day 3 of storage, which remained the same until day 5. The highest *P. fluorescens* growth population was recorded at day 7. Growth of *P. fluorescens* increased by 4 log during the storage period of 7 days. APC remained relatively constant, with a significant increase ($p<0.05$) between the counts enumerated at day 0 compared to day 5 and day 7. Similar levels of *E. coli* O157:H7 and *P. fluorescens* were recovered on day 3. *P. fluorescens* counts on days 5 and 6 were higher than *E. coli* O157:H7 counts during the same period.

Growth of *E. coli* O157:H7 showed a 2 day lag phase when combined with 10^6 $\log_{10}$ cfu/ml *P. fluorescens* inoculum (Fig. 1b). By day 3, growth increased significantly ($p<0.05$) compared to start of the storage period. The highest *E. coli* O157:H7 growth population was achieved from day 5 and maintained until the end of the experiment. The growth of *E. coli* O157:H7 under both experimental environments (*P. fluorescens* inoculation levels 10^2 or 10^6 $\log_{10}$ cfu/ml) was similar by day 7 indicating that the level of *P. fluorescens* did not affect the growth of *E. coli* O157:H7.

The growth of *P. fluorescens* (Fig 1b) showed a similar pattern as *E. coli* O157:H7 at the beginning of the storage period with a 2-day lag phase. By day 3, there was a significant ($p<0.05$) increase in growth, which remained at the same level until day 5. A significant ($p<0.05$) increase in *P. fluorescens* growth was observed at day 7. *P. fluorescens* increased by 3 log over the storage period. APC followed a similar trend to *P. fluorescens* (Fig 1b).

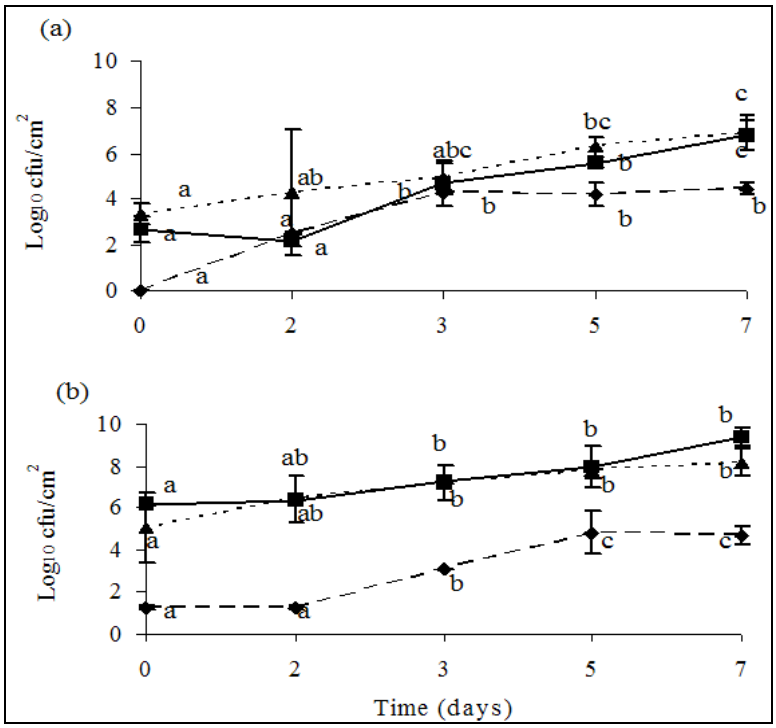

Figure 1. Effect of inoculation levels of *P. fluorescens* (10^2 and 10^6) on the growth of inoculated *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml) on sterile beef (n=60), aerobically packaged and stored for 7 days at 4 °C. (a) Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^2 $\log_{10}$ cfu/ml *P. fluorescens* in the inoculum; (b) Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^6 $\log_{10}$ cfu/ml *P. fluorescens* in the inoculum. Means for the same bacterium ♦ *E. coli* O157:H7 ■ *P. fluorescens* ▲ APC with different letter notations are significantly different at $p<0.05$.

Effect of different levels of *E. coli* O157:H7 and *L. plantarum* in the inoculum on the growth of *E. coli* O157:H7 on beef loins

The level of *L. plantarum* in the inoculum and the storage time significantly ($p<0.05$) affected the survival and growth of *E. coli* O157:H7 and *L. plantarum* on beef pieces, while both factors did not affect APC ($p=0.37$). The onset of *E. coli* O157:H7 inhibition was also affected by the level of *L. plantarum* in the inoculum.

When the levels of *E. coli* O157:H7 and *L. plantarum* were similar (10^2 $\log_{10}$ cfu/ml) in the inoculum suspension (Figure 2a), *L. plantarum* did not show immediate inhibition of

E. coli O157:H7. There was a similar numerical increase in the *E. coli* O157:H7 count recorded at week 1 and week 2. The highest growth population of *E. coli* O157:H7 was determined at week 3 after which no more growth occurred. Instead, the *E. coli* O157:H7 population declined, on average, by 2 log by week 4 to 2.03 $\log_{10}$ cfu/cm^2. The decline in the *E. coli* O157:H7 population continued up to week 5 and was too low to detect by week 6.

Similar to *E. coli* O157:H7, *L. plantarum* counts remained stable from the beginning of storage to week 1. There was a significant ($p<0.05$) increase recorded at week 2. At week 3, the *L. plantarum* growth curve showed 1.11 log increase compared to week 2. From week 3, *L. plantarum* counts remained similar until week 7, indicating a 5 week long stationary phase, which was followed by a 1.4 log decline recorded at week 8, indicating the beginning of the death phase of *L. plantarum*. APC showed an increase by week 1 that continued until week 3. APC then remained constant for the remainder of the storage time.

When a lower level of *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml) was combined with a higher level of *L. plantarum* (10^4 $\log_{10}$ cfu/ml) in the inoculum (Fig. 2b), the adverse effect of *L. plantarum* on the growth and survival of *E. coli* O157:H7 was recorded earlier compared to the onset of such an effect when the same levels of *E. coli* O157:H7 and *L. plantarum* in the inoculum suspension. Low levels of *E. coli* O157:H7 survivors were detected at week 1 and week 2. From week 3 onwards, *E. coli* O157:H7 was not recovered from beef loin pieces. The *L. plantarum* growth curve showed significant growth of the organism by week 2), which remained at the same level by the end of the experiment. However, the count of *L. plantarum* at week 4 was significantly higher ($p<0.05$) compared to that recorded at week 2. APC were relatively constant throughout the storage period. APC recorded during week 8 was significantly higher ($p<0.05$) compared to weeks 0 and 1. *L. plantarum* count increased by 2 log when comparing the starting count and the count at the end of storage.

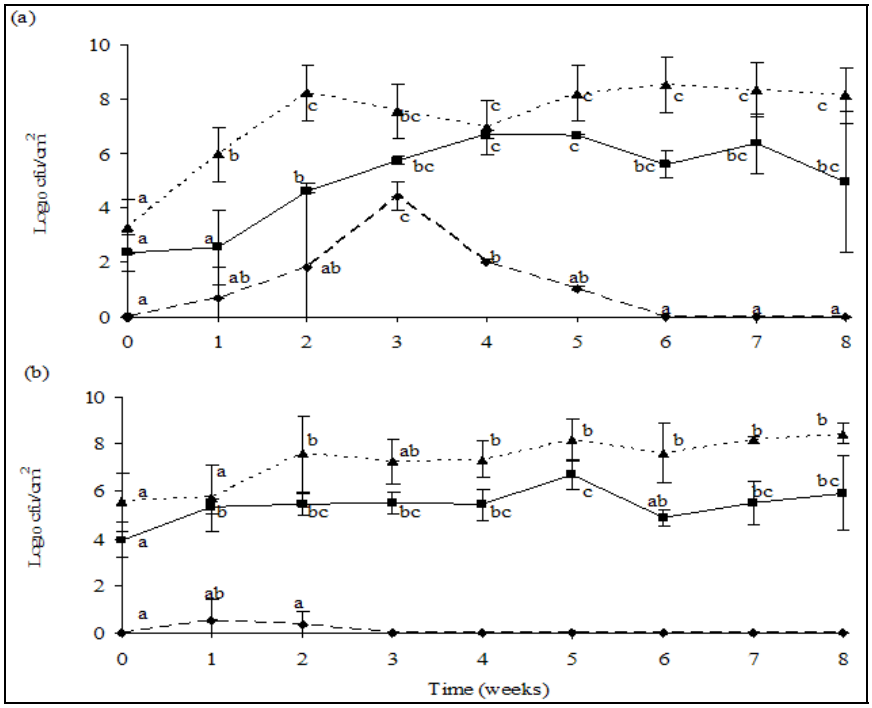

Figure 2. Effect of inoculation levels of *L. plantarum* (10^2 and 10^4 $\log_{10}$ cfu/ml) on the growth of inoculated *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml) on sterile beef (n=108), vacuum packaged and stored for 8 weeks at 4 °C. (a) Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^2 $\log_{10}$ cfu/ml *L. plantarum* in the inoculum; (b) Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^4 $\log_{10}$ cfu/ml *L. plantarum* in the inoculum. Means for the same bacterium, with different letter notations ♦ *E. coli* O157:H7 ■ *L. plantarum* ▲ APC are significantly different at $p<0.05$.

DISCUSSION

Effect of different levels of *E. coli* O157:H7 and *P. fluorescens* in the inoculum on the growth of *E. coli* O157:H7 on beef loins

The application of a constant level of *E. coli* O157:H7 with either a similar or a higher level of *P. fluorescens* in the inoculum showed interesting growth patterns for both bacteria, as well as the APC. *E. coli* O157:H7 could not be quantified immediately after inoculation at day 0, while *P. fluorescens* was detected. The inability to recover *E. coli* O157:H7 from inoculated samples is in agreement with Thran *et al.* (27) who did not detect *E. coli* O157:H7 in rumen contents when testing directly within 24h of storage. Detection was only achieved after enrichment. The results of this study, showed that 18h cultures of *E. coli* O157:H7 and *P. fluorescens* required 2 days to adapt and start growing on meat stored aerobically at 4 °C.

By day 3, under similar inoculation levels, the growth populations of both organisms had increased significantly, implying that there was no antagonism between these organisms before day 3. An antagonistic interaction between *E. coli* O157:H7 and *P. fluorescens* was observed after day 3, wherein, *E. coli* O157:H7 stopped growing, while *P. fluorescens* continued to grow and reached its maximum growth density at day 7. The retardation of *E. coli* O157:H7 might be due to the effects of the available nutrients on meat, the suppression by background *P. fluorescens* or the combined effect from both nutrients and *P. fluorescens.* Tsigarida *et al.* (28) reported an accelerated consumption of glucose by pseudomonads when co-cultured with other bacteria. Free glucose is a minor component of meat, 0.1 to 0.5 % (10, 20). Therefore, *Pseudomonas* species have a competitive advantage over other organisms due to their ability to transform glucose rapidly to gluconate in chilled muscle foods stored aerobically (6). The presence of glucose enhances the inhibition of *E. coli* O157:H7 by *Pseudomonas* spp., particularly at low storage temperatures (23). In the absence of glucose, the inhibition of *E. coli* O157:H7 was weak at temperatures above 10 ºC, while at 25 ºC, there was minimal inhibition of the pathogen, irrespective of glucose (23).

Growth of bacteria on the surface of meat depends on the rate of diffusion of fermentable substrates from within the meat to the surface (10). When the rate of transfer of such substrates slows down the rate of growth declines to the point where the rate at which the substrates become available is only sufficient for cell maintenance and not growth. Therefore, the cessation in growth between days 3 and 5 could signify the period that *P. fluorescens* had to change their metabolism to utilize amino acids and lactic acid upon the depletion of glucose (10). Furthermore, Pseudomonads synthesize siderophores that function to sequester available iron (16). Therefore, *P. fluorescens* could also have exerted a siderophore inhibitory effect (5), on *E. coli* O157:H7, resulting in levels of the latter not increasing after 3 days of storage at 4 °C. The APC curve showed a similar pattern to that of *P. fluorescens,* confirming that the *P. fluorescens* cells contributed mostly to the APC of the sterilized beef loin samples, as would have been expected.

When beef pieces were inoculated with a higher level (10^6 $\log_{10}$ cfu/ml) of *P. fluorescens*, *P. fluorescens* showed a slower growth rate, unlike when similar levels were used, which could probably be due to competition among *P. fluorescens* for growth space on the limited growth area. *E. coli* O157:H7 continued to grow in the presence of *P. fluorescens,* even when the levels of *P. fluorescens* had increased to 10^7 $\log_{10}$ cfu/cm^2, a level attributed to slime and off-flavours formation (20). Therefore, although *E. coli* O157:H7 survived until the end of the experiment, beef loins would have been rejected based on appearance.

The continued increase in *E. coli* O157:H7 growth population between days 3 and 5 demonstrates that *E. coli* O157:H7 competes well with *P. fluorescens* on meat. APC growth curve was similar to that observed when the levels of *E. coli* O157:H7 and *P. fluorescens* were similar. In this study, both inocula levels of *P. fluorescens* did not inhibit the growth of *E. coli* O157:H7 on refrigerated beef loins. The level of *P. fluorescens* does not inhibit growth of *E. coli* O157:H7 on

aerobically stored beef. *E. coli* O157:H7 survived at levels that can cause food-borne illness regardless of the level of *P. fluorescens* on meat. Comprehensive control strategies at primary production and slaughtering levels are required to eliminate the contamination of meat and meat products with *E. coli* O157:H7.

Effect different levels of *E. coli* O157:H7 and *L. plantarum* in the inoculum on the growth of *E. coli* O157:H7on beef loins

When beef pieces were inoculated with similar levels of *E. coli* O157:H7 and *L. plantarum, E. coli* O157:H7 growth was only determined after 3 weeks of storage. The ability of *E. coli* O157:H7 to grow under acidic conditions could be as a result of acid tolerance. *E. coli* O157:H7 acquired increased acid tolerance after being incubated in acidic washings of sublethal pH at 4 °C (26). Likewise, in this study during the lag phase, the pH of beef could have decreased due to *L. plantarum* activities, exposing *E. coli* O157:H7 cells to acidic conditions. The cells then become adapted to acidic conditions and continued to grow, hence the exponential cell growth by week 3. In support of the findings of this study, Dykes *et al*. (7) concluded that *E. coli* O157:H7, unlike generic *E. coli* was less inhibited by the drop in pH. Low temperature and acidification enhanced the acid tolerance of *E. coli* O157:H7 on vacuum packaged beef (7).

L. plantarum produces plantaricin, a bacteriocin with inhibitory activity towards both Gram positive and Gram negative bacteria, including food pathogens (*Listeria*, *Staphylococcus* and *Salmonella*) (15, 8, 9, 17, 18). In this study, as soon as *L. plantarum* cells entered the stationary growth phase, *E. coli* O157:H7 counts showed a steady decline in growth. After week 6, *E. coli* O157:H7 was not recovered from beef samples. The inhibition of *E. coli* O157:H7 growth could be attributed to the effect of plantaricins produced by *L. plantarum* after week 3. This observation corresponds to the reported production of bacteriocins by *L. plantarum* when its growth transcends from exponential to stationary phase (4, 11).

The growth curve of *L. plantarum* showed that *L. plantarum* cells required 1 week before growth could be detected. Similarly, in another study, LAB cells were only detected from vacuum packaged goat minced meat after a 9 day storage period at 4 °C (3). The inability of *L. plantarum* to grow exponentially could be due to the storage temperature of 4 °C. This is in keeping with the findings of Paynter *et al*. (21) who found that *L. plantarum* did not grow at 4 °C for 56 days, while growth occurred at higher incubation temperatures from 20 to 37 °C. As expected, the APC growth curve showed cells entering the stationary growth phase from week 2 onwards until the end of the experiment, a similar trend to *L. plantarum.*

Unlike with similar levels of *E. coli* O157:H7 and *L. plantarum,* the inhibition of *E. coli* O157:H7 at the higher inoculation level (10^4 $\log_{10}$ cfu/ml) occurred earlier. There was insignificant *E. coli* O157:H7 growth initially and the organism was not recovered at all from meat samples from week 2 onwards. The earlier inhibition of *E. coli* O157:H7 corresponded to the earlier entry of *L. plantarum* cells into the stationary phase, signifying a possible earlier onset of the production of plantaricins. This result shows that plantaricins, lower storage temperature (4 °C), higher level of *L. plantarum* in the inoculum and vacuum packaging had a combined inhibitory effect on the growth of *E. coli* O157:H7 on beef.

On vacuum packaged beef, *L. plantarum* culture treatment is beneficial in inhibiting the survival and growth of *E. coli* O157:H7. The higher the cell suspension of *L. plantarum*, the earlier the onset of the inhibition of *E. coli* O157:H7. Further studies to test the application of *L. plantarum* as a bio preservation technology are needed.

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