Salmonella and E. coli are the most important genera in meat (Devlieghere et al., 2004). Salmonella is related with 93.8 million of worldwide human infection cases, and 155,000 annually deaths (Majowicz et al., 2010). In the Salmonella infections outbreaks, beef is the most commonly implicated food (Jackson, 2014). In the same way, E. coli is the most frequent bacteria isolated from meat and its used as indicator of fecal contamination, their pathogenic serotypes (enterohemorrhagic, enteropathogenic, diarrheagenic and verotoxigenic) are responsible for multiple outbreaks of food-borne diseases throughout the world (Hiko et al., 2008), only in USA is attributed about 40,000 annually deaths, and ground beef is commonly food vector (Russo and Johnson 2003).

The natural microbiota (NM) of meat is a complex ecosystem involving several types of microorganisms, including some LAB populations with inhibitory capacity. Over time, the use of LAB to preserve meat has been proposed, however, most of the suggest bacteria are from fermented products or ATCC strains. Therefore, the objective of this work was to isolate meat native LAB strains from ground beef with antagonistic capacity against two reference bacteria (Salmonella and E. coli) and estimate the inhibition effect of LAB cultures and their CFS, using in vitro techniques.

Fifty samples of raw ground beef were aseptically obtained according to Official Mexican Standard No. 109, from butchers and meat retailers in Texcoco de Mora, Mexico, and which were transported and stored (-20 °C) in the laboratory of Livestock Microbiology, Chapingo Autonomous University. Aliquots (25 g) of each sample were placed on sterile bottles and homogenized with peptone solution (225 mL, Bioxon) in a blender for 1 min. Serial dilutions were made with sodium chloride solution 0.85% w/v (saline) and pouring (200 µL) in de Man, Rogosa and Sharpe (MRS, Dibico) agar and incubated 48 h at 37 °C. LAB characteristic colonies were taken and isolated by streaking in MRS agar. All bacteria strains were stored in appropriate media with glycerol (30%) at -20 °C.

In this study, lactic acid bacteria (LAB) strains were isolated from ground beef, and it was analyzed if they have any effect on the growth of two reference bacteria (Salmonella sp. and Escherichia coli). It was found that five isolates showed an inhibitory effect in both reference bacteria by spot at the lawn assay. These bacteria were selected to perform growth kinetics in co-culture to determine if they modify the growth parameters of the reference bacteria. Subsequently, LAB cultures and three treatments (crude extract, thermally treated and thermally treated with neutral pH) of cells free supernatants (CFS) were screened by the agar well diffusion assay. In co-culture, selected LAB altered the growth rate and reduce the maximum population of both reference bacteria. While, LAB cultures and CFS also showed antimicrobial activity, and there was no significant difference among CFS treatments. LAB isolated from ground beef showed an antimicrobial effect against the reference bacteria that could be used for meat biopreservation purposes.

Key words: Biopreservation / inhibition / pathogenic / Salmonella / Escherichia coli.
Inhibition assay of bacteria were performed by spot on the lawn assay (Tagg and McGiven, 1971), conserved strains were activated by streak on agar medium and incubating for 24 h at 37 °C and were verified their purity. LAB were incubated in MRS broth over night at 37 °C, the culture was diluted with saline solution until it had a turbidity of 4 on the McFarland scale. Indicator bacteria (Salmonella and E. coli) were spread over Muller-Hinton (Dibico) agar Petri dish, and LAB were spotted (10 µL) onto surface. The dish was incubated until indicator bacteria lawn was visible. The LAB those that had an inhibition zone around the spot, which indicates antimicrobial activity, were selected and stored (Shokryazdan et al., 2014).

Five strains which can show antimicrobial activity for both reference bacteria were selected and identified by API 50 CH (Biomerieux). These LAB were activated and streaked in MRS agar plate and incubated 24h at 37 °C. Good growth colonies were taken and cultured in MRS broth at 37 °C until turbidity were 4 of McFarland. Cultures were bated in a boiling water for 5 min, the pH was neutralized with NaOH (0.1 M) and centrifuged at 10,000 rpm for 10 min at 4 °C, the resulting supernatants were stored at -20 °C. The antimicrobial activity of cells-free supernatants (CFS), heat treated (CFSH) and heat treated and neutralized pH (7.0, ± 0.5) (CFSHN) were tested by agar wells diffusion assay (NCCLS, 1993). 200 µL of activated culture in brain heart infusion (BHI) (4 of McFarland Scale, at 37 °C) of each reference bacterium was pouring on plate with Mueller-Hinton agar (Dibico), wells diameter was 3 mm, and plates was incubated 24 h at 37 °C. The inhibition zone diameter (IZD) were expressed in arbitrary unit per milliliter (AU/mL) and were measured using the following formula.

\[
\text{AU/mL} = \frac{\text{IZD} \times 1000}{\text{Volume in the well (µL)}}
\]

Bacterial growth kinetics of the reference bacteria were carried out in monoculture and in co-culture with LAB, this was done by activating the bacteria by streaking in agar plates with appropriate medium, MRS for LAB and nutritive agar for reference bacteria (E. coli and Salmonella), and incubated 24 h at 37 °C, for activation. After, well-grown colonies were taken and incubated in appropriate broth, MRS broth for LAB and BHI for reference bacteria and incubated at 37 °C until turbidity was as 4 McFarland scale. 1 mL of culture was washed trice with saline by centrifugation at 10,000 rpm at 4 °C for 15 min, harvested bacteria were resuspended in 1 mL of saline. Subsequently, they were inoculated, at 1:1 ratio (LAB: Reference bacteria), in BHI and incubated at 37 °C.

The bacterial count was carried out by pouring on plate method, taking 1 mL of co-culture each count time (0, 2, 4, 8, 10, 18, 24, 36 and 48 h). Culture medium were depending on kind of bacteria was count, Xylose Lysine Deoxycholate agar (XLD) for 24 h at 42 °C, for Salmonella; and Eosin and Methylene Blue agar (EMB) for 24h at 37 °C, for E. coli. The adjustment of growth curves and parameters calculation were made using the new logistic model (NLM) (Fujikawa, 2010).

\[
\frac{dN}{dt} = rN\{1 - (\frac{N}{N_{\text{max}}})^m\}\{1 - (\frac{N_{\text{min}}}{N})^n\}
\]

Where:
- \(t\) = time
- \(N\) = is the population at time \(t\)
- \(r\) = is rate constant of growth
- \(N_{\text{max}}\) = is the maximum population
- \(N_{\text{min}}\) = is the minimal population
- \(m\) = is a deceleration curvature parameter
- \(n\) = is lag phase parameter

The variables of growth kinetics and arbitrary units were analyzed using a totally random design, based on general linear model in the SAS statistical software (9.4, 2014). Each treatment had two repetitions. The averages of treatment were analyzed using Tukey’s multiple comparisons test, and results were considered significant when \(P<0.05\).

Total of isolated LAB strains (115) were screened for antagonism activity, 44 had an inhibitory effect against at least one reference bacterium, of which, five strains inhibited the growth of both bacteria. Its identification was performed by API 50 CH kit and apiweb tool (Biomerieux) (Table 1).

| Isolate | Genus and species                        |
|---------|----------------------------------------|
| B9      | Lactobacillus delbrueckii              |
| B27     | Lactococcus lactis<sup>a</sup>         |
| B29     | Lactobacillus fermentum                |
| B40     | Lactococcus lactis<sup>b</sup>         |
| B44     | Leuconostoc mesenteroides              |

Different letters indicate different strain.

The maximum growth \(N_{\text{max}}\), rate of constant growth \((r)\) and lag phase \((\text{lag})\) differed \((P<0.05)\) among selected LAB strains (Table 2). The highest \(N_{\text{max}}\) was
TABLE 2. Mean values of growth parameters at 48h, MRS broth.

| LAB strain | B9          | B27       | B29       | B40       | B44       |
|------------|-------------|-----------|-----------|-----------|-----------|
| r          | 0.97±0.03   | 0.98±0.01 | 1.05±0.06 | 0.81±0.01 | 0.45±0.05 |
| lag        | 0.28±0.19   | 0.44±0.3  | 1.26±0.26 | 0.52±0.32 | 0.58±0.98 |
| Nmax       | 7.82±0.01   | 7.59±0.02 | 7.01±0.01 | 6.11±0.02 | 6.46±0.01 |
| pH         | 4.14±0.001  | 4.57±0.01 | 4.18±0.01 | 4.15±0.002| 4.34±0.001|

Means±SD: Means and standard deviation.
Different letters in the lines indicate a statistically significant difference (Tukey test, P<0.05).

r = rate of constant growth
lag = lag phase
Nmax = maximum cell population (log CFU/mL)
pH = potential of hydrogen

corresponding to B9 (7.82 log CFU/mL) followed by B27 and B29 (7.59 and 7.01 log CFU/mL), and, B40 and B44 were the lowest (6.11 y 6.46 log CFU/mL).

Similar to Nmax, the highest rate of constant growth (r) (P<0.05) were recorded in B9, B27 and B29 (0.97, 0.98 and 1.05) followed by B40 (0.81). Meanwhile, B44 had the lowest r (0.024) (P<0.05). In the other hand, B9 required a longer period of time (lag) to initiate the growth (4.6 h) (P<0.05) with respect to B44, B40, B27 and B9 treatments (Table 2). Finally, the lowest pH was observed in B9 and B40.

Most of growth kinetic parameters of Salmonella were different (P<0.05) compared when its growing in monoculture and its growing in co-culture with each selected LAB (Table 3). Growth rate (r) was not different (P>0.05) between Salmonella monoculture and B9 (0.96 and 0.95), but r was lower in B40 (0.93) followed by B27, B29 and B40 (0.63, 0.70, 0.69). Meanwhile, lowest lag phase was recorded in B27 (0.35) followed by B29, B40, B44 and B9. Moreover, the Nmax in all LAB co-cultures was lower (P<0.05) than Salmonella monoculture, lowest were found in B29, B40 and B44 co-cultures. Graphically, it is observed that the reduction in Salmonella Nmax in co-culture was greater since the

tenth hour (Fig. 1).

On the other hand, r of E. coli was similar (P>0.05) to B27 and B44 (0.77 and 0.81), and different in B40 followed by B9 and B29 (Table 4). Meanwhile, shortest lag phases were recorder in B40 and B9 and longest in B27, B29, B44 and E. coli. However, Nmax was lower (P<0.05) in LAB co-cultures than E. coli monoculture,
lowest was found in B9, similar to B40 and followed by B29, B44 and B27. Fig. 2 shows that the reduction of E. coli $N_{\text{max}}$ in co-culture with LAB occurs after the tenth hour.

The mean values of LAB culture inhibition zone diameter (IZD) are shown in Table 5. It is observed that the highest IZD in Salmonella was produced by B40 LAB strain (367.2 AU/mL) followed by B29, B9, B27 and the lowest was B44. On the other hand, for E. coli, the highest IZD was produced by B9 (209.8 AU/mL) followed by B29, B40, B44 and B27.

Similarly, the IZD of LAB CFS showed an antagonistic effect, for Salmonella, the greatest effect was recorded by B40, followed by B29 and the lowest by B27 and B29. While for E. coli the greatest effect was observed by B9 and to a lesser extent and without difference among them B27, B29, B40 and B44. On the other hand, no significant differences ($P>0.05$) were found between the different CFS treatments. The results are shown in Table 6.

In this work, different isolations of ground beef LAB were found, which had an antagonistic effect against the reference bacteria. The five selected strains were inhibiting the growth of both reference bacteria. However, the effect was not the same towards the reference bacteria, if not it was always greater towards any of them.

### Table 4. E. coli kinetics parameters in monoculture and co-culture with LAB, BHI broth.

|        | B9        | B27        | B29        | B40        | B44        |
|--------|-----------|------------|------------|------------|------------|
| $r$    | 0.60±0.02b| 0.77±0.002a| 0.63±0.011c| 0.69±0.01b | 0.81±0.003a|
| $\text{lag}$ | 0.76±0.270a| 0.62±0.200d| 0.71±0.041b| 0.40±0.160b| 0.02±0.06b |
| $N_{\text{max}}$ | 7.91±0.061a| 5.56±0.160d| 6.86±0.021b| 6.01±0.008b| 5.79±0.020a|

Mean±SD: Means and standard deviation.
Different letters in the lines indicate a statistically significant difference (Tukey test, $P<0.05$).

$r$: rate of constant growth

$\text{lag}$: lag phase

$N_{\text{max}}$: maximum cell population (log CFU/mL)

### Table 5. Mean values of inhibition zone diameters (AU/mL) of LAB culture, agar well diffusion assay.

|        | B9        | B27        | B29        | B40        | B44        |
|--------|-----------|------------|------------|------------|------------|
| Salmonella | 208.1±1.1bc| 182±7.4cd | 213.2±7.2b | 367.2±4.5a| 161.2±7.3d|
| E. coli  | 209.8±0.35a| 155±7.1b  | 165.1±7.1b | 160.1±14b | 160±14.1b |

Mean±SD: Means and standard deviation.
Different letters in the lines indicate a statistically significant difference (Tukey test, $P<0.05$).

### Table 6. Means values of inhibition zone diameters (AU/mL) of cell-free supernatant treatments by agar wells diffusion assay.

|        | B9        | B27        | B29        | B40        | B44        |
|--------|-----------|------------|------------|------------|------------|
| Salmonella | CFS 203.56±3.39cd | 178.36±7.21cd | 208.94±7.21b | 360.86±5.851a| 157.98±7.22a|
|         | CFSH 207.11±4.05b | 178.72±7.22cd | 209.35±7.21b | 348.98±11.96a| 158.29±7.23a|
|         | CFSHN 207.26±7.14b | 176.93±7.15cd | 207.26±7.15b | 350.86±4.230a| 156.71±7.14d|
| E. coli  | CFS 212.04±2.81a | 157.98±7.21b  | 168.17±7.20b | 170.57±3.81b | 163.07±14.41b|
|         | CFSH 216.91±3.46d | 158.29±7.22b  | 168.50±7.20b | 170.90±3.84b | 163.40±14.31b|
|         | CFSHN 209.27±4.31a | 156.71±7.16b  | 166.82±7.15b | 161.76±14.3b | 161.76±14.01b|

Mean±SD: Means and standard deviation.
Means with different letters in the same column are significantly different ($P<0.05$).

CFS = cells-free supernatant.

CFSH = heat treated cells-free supernatant.

CFSHN = heat treated and pH neutralized cells-free supernatant.
In order for LABs with inhibitory potential to efficiently colonize the environment, it is desirable that they have desirable characteristics such as a good growth rate, a short lag phase, a maximum high population as well as a greater pH reduction (Zaher and Fujikawa, 2011); of the isolated strains Lb. delbrueckii (B9) record the best parameters.

In the co-culture with LAB it’s found a reduction of the maximum population, for Salmonella Lb. fermentum (B29), L. lactis (B40) and L. mesenteroides (B44) were the strains that reduced it the most, while E. coli was the most reduced by Lb. delbrueckii and L. lactis. The reduction of maximum population could be due to a nutrient competition effect (Fujikawa et al., 2014). In addition, the reduction of the pH of the medium could contribute to the inhibition of the growth of reference bacteria, that is congruent because Lb. delbrueckii and L. lactis were the strains that reduced the pH (Ji-yeon et al., 2015). However, these results differ with those recorded by the agar well diffusion assay where the strains that produced a wider IZD were for Salmonella, L. lactis and for E. coli, Lb. delbrueckii, this inhibitory activity is similar to that recorded by their CFS. This indicates that these bacteria they produce some antimicrobial substance found in the supernatant (Angmo et al., 2016; Yan and Lee, 1997). Also, because there were no differences when the pH was neutralized, lactic acid can be ruled out as the main inhibitory agent (Favaro and Todorov, 2017; Ji-yeon et al., 2015). In addition, the inhibitory substance is heat-resistant because there was no difference when heat treated. These antimicrobial substances can be such as reuterine, surfactants, bacteriocins and bacteriocins-like (Alegría et al., 2010; Hugas, 1998) these are produced during the lag phase and, besides affecting pathogens they also affect spoilage bacteria (Jones et al., 2008; Stiles, 1996). On the other hand, the results of CFS as well as the thermally treated and with neutral pH, were weak, this differs from other works (Alegría et al., 2010; Yan and Lee, 1997), mainly because in other works a semi-purification process was carried out that increased the concentration of the inhibitory substance. Despite this, antagonistic CFS activity was observed and in co-culture it was also reduced by at least 1.5 log units.

Lb. delbrueckii showed a moderate lag phase, the lowest pH and a high growth rate and $N_{max}$. In addition, in co-culture it showed greater effectiveness against E. coli than against Salmonella. In the same way, its CFS had an adequate inhibitory effect, which could indicate bacteriocin-like substance (Simova et al., 2008).

On the other hand, L. lactis was the LAB isolated from meat that had better behavior, its growth was moderately fast, and its maximum population was the lowest, however as regards the activity of its CFS was higher in Salmonella than against E. coli, the same was observed in co-culture, the reports also indicate the ability to produce lacticin (Mirkovic et al., 2016). Among the most prominent genres to be used in biopreservation protocols, those that have the capacity to produce bacteriocins stand out, in addition they are required not to produce spoilage (Porto et al., 2017). In the industry already has the use of bacteriocins to sanitize the surface of meat products such as sausages, in view of this L. lactis subsp. lactis isolated from the meat could be used for this purpose (Ünlü et al., 2016).

The consumer reluctance to purchase products with chemical preservatives results in use of alternatives (Djadouni and Kihal, 2012). Use of bacteriocins raises the same ethical question, as it would be use of substances foreign to food. However, it must be borne in mind that various types and quantities of bacteriocins produced by LAB are already consumed in foods such as dairy and fermented meat. To date there have been no reported toxicity problems but some cases of hypersensitivity or allergy (Abdel-Mohsein et al., 2010).

LAB strains isolated in this work had modifying the growth kinetics parameters and producing inhibitory effects against the reference bacteria (Salmonella and E. coli). Also, the inhibitory effect of the selected bacteria was similar between co-culture and CFS, it would indicate that the antagonistic action is the result of the production of some antimicrobial agent such as a bacteriocin.

Thus, the natural microbiota of meat is a source of antibacterial LAB strains. Lactococcus lactis and Lactobacillus delbrueckii, isolated in this work, showed the greatest inhibitory effect against reference bacteria, respectively, and have the potential to be used in biopreservation protocols, to inhibit the growth of pathogens and improve the shelf life food. However, it is
required to test these strains, to confirm if their antagonistic effect is the same in food conditions.

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