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In Vitro evaluation of antimicrobial activity of Lactobacillus acidophilus against some pathogens

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A B S T R A C T

Bacterial infections cause severe losses in poultry farming. Clostridium perfringens and Escherichia- coli are two major pathogenic bacteria readily found in the broiler environment. They cause high- morbidity and mortality in poultry worldwide because of necrotic enteritis and colibacillosis, respectively. Furthermore, Staphylococcus aureus is an important cause of omphalitis, bumble foot and gangrenous dermatitis. Antimicrobial resistance is one of the most serious global public health threats that necessitates alternative strategies. Probiotics have been emerging as a safe and effective alternative to antibiotics. Lactobacillus being the most used probiotic. Therefore, here the antimicrobial activity of Lactobacillus acidophilus against some pathogens namely Clostridium perfringens, Staphylococcus aureus and Escherichia coli were tested. The antibacterial effect of L. acidophilus was investigated by time kill assay, agar well diffusion and agar spot test. The obtained results showed that L. acidophilus had strong antimicrobial activity against the three bacterial pathogens. Moreover, L. acidophilus exhibited a strong auto-aggregation phenotype and marked coaggregation with Cl. perfringens. In addition, inhibition of gas production from Cl. perfringens by L. acidophilus was evaluated. Alpha-toxin has been implicated as one of the major virulence factors of Cl. perfringens inducing avian necrotic enteritis. To investigate whether alpha toxin have adverse effects on L. acidophilus, viability assay proved that L. acidophilus was not remarkably affected by incubation with different concentrations of alpha toxin and different incubation time. Taken together, our results suggest that L. acidophilus exhibits strong inhibitory effects against Cl. perfringens, E. coli and S. aureus, and has strong co-aggregation abilities. However, further in vivo investigations are required.

Keywords: L. acidophilus, Cl. perfringens, S. aureus, E. coli, antimicrobial activity, aggregation, alpha toxin.

1. Introduction

Poultry production has undergone a substantial increase compared to other animal food-producing industries (Yegani and Korver, 2008). The poultry industry faces challenges, especially with the ban of using antibiotics as growth promotors because of the risk of elevated-antimicrobial resistance (Huyghebaert et al. 2011). Therefore, there is a need to use probiotics that can improve the economic indices and resistance to bacterial pathogens (Aazami et al. 2014, Cean et al. 2015). Lactobacilli have been widely used as probiotics in the poultry industry (Aazami et al. 2014) constituting an important part of the natural microbiota and as a potent interfering bacterium displaying several defense mechanisms against some pathogens (Gorska et al. 2016). Clostridium perfringens plays an important role in the etiology of necrotic enteritis (NE), which is the cause of great economic losses in the poultry production industry (Cooper et al. 2009). Cl. perfringens type A produce Alpha toxin, which is considered one of the most virulence factors inducing clinical and subclinical avian NE) Coursodon et al. 2010, M'Sadeq et al. 2015).

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Avian pathogenic Escherichia coli cause colibacillosis in poultry that includes systemic and localized infections. The localized infections are omphalitis, swollen head syndrome, cellulitis, and diarrhea. Whereas systemic infections include respiratory colisepticemia, enteric colisepticemia, and neonatal colisepticemia (Ewers et al. 2003). Staphylococcus aureus treatment remains challenging to manage due to the emergence of multi-drug resistant strains and very potent biofilm-producers resulting in high morbidity, high mortality, and increased treatment costs (Gardete and Tomasz, 2014). The bacterial interactions between L. acidophilus and pathogenic bacteria could offer potential novel therapeutic approaches to combat pathogens (Spurbeck and Arvidson, 2010, Yu et al. 2013). Microorganisms directly compete with each other by producing various antimicrobial compounds, including bacteriolytic enzymes, bacteriocins or biosurfactants that may also change the physical and chemical conditions of the surrounding environment (Merk et al. 2005). Evaluation of antimicrobial activity of L. acidophilus against Cl. perfringens type A, Escherichia coli and Staphylococcus aureus by several techniques as time kill, agar well diffusion and agar spot technique (Anas et al. 2008, Do Carmo et al. 2016), in addition to gas inhibition and coaagregation assay for Cl. perfringens (Collado et al. 2008, Golic et al. 2017) were used to explain antimicrobial activity of L. acidophilus. Coaggregation is a highly specific recognition and adhesion of genetically distinct bacteria. It is mediated by complementary protein adhesins and polysaccharide receptors on the cell surface of coaggregating cells (Rickard et al. 2003). This phenomenon is distinct from autoaggregation, which is the recognition and adhesion of genetically identical bacteria (Khemaleelakul et al. 2006). Autoaggregation assay achieves an adequate mass to form bacterial biofilms, changes in biofilm architecture and altered species composition of biofilms (Kolenbrander et al. 2006, Hojo et al. 2009). Furthermore, Coaggregation between L. acidophilus and pathogens may constitute an important host defense mechanism against infection. Therefore, in the present study, the antimicrobial activity and aggregative abilities of L. acidophilus against Cl. perfringens, E. coli and S. aureus were evaluated.

2. Materials and Method

2.1. Bacteria

Lactobacillus acidophilus and three pathogenic strains (Clostridium perfringens, Staphylococcus- aureus and Escherichia coli) were used in this study, they were kindly provided by the Animal Health Research Institute in Dokki and Alexandria Provincial Lab, Egypt. Alpha toxin of Cl. perfringens was kindly provided by Sera and Bacterial Vaccines Institute, Abbasia, Egypt. The identity of the strains was confirmed by biochemical tests (Cruickshank et al. 1975, Holt et al. 1994, Quinn et al. 2002), Matrix-assisted laser desorption/ionization (MALDI) (Anderson et al. 2014, Chean et al. 2014) and scanning electron microscope (SEM) for both L. acidophilus and Cl. perfringens (Nation, 1983).

Lactobacilli were grown in de Man, Rogosa, Sharpe (MRS) broth (Oxoid, UK) under anaerobic- conditions at 37ºC for 24 h (Holt et al. 1994). E. coli was cultured aerobically in brain-heart infusion (BHI) broth (Oxoid) then Eosin methylene blue agar (EMB) (Oxoid) at 37 °C for 18 h (Quinn

et al. 2002). Cl. perfringens was cultured in Reinforced Clostridial medium (RCM) then in thioglycolate medium and incubated at 37 ºC for 24–48 h under anaerobic atmosphere (Cruickshank et al. 1975). S. aureus was cultivated in mannitol salt agar (Oxoid) and brain-heart infusion medium (Oxoid) under aerobic conditions at 37 ºC for 24 h (Quinn et al. 2002). Each strain was cultivated individually.

2.2. Preparation of bacterial inoculum

The cell density was determined using 0.5 McFarland standard of Barium chloride solution (Valgas et al. 2007) as follows: a loopful of 24 h anaerobically surface growth on MRS agar for L. acidophilus, tryptose sulphite cycloserine (TSC) agar for Cl. perfringens and a loopful of aerobically 24 h surface growth on nutrient agar slopes for E. coli and S. aureus, were transferred individually to 5 ml of physiological solution till moderate turbidity was developed to match 0.5 McFarland standard corresponding to cell density approximately of 108 CFU, according to WHO (1993).

2.3. Preparation of neutralized cell-free culture supernatants (CFCS) of Lactobacilli

L. acidophilus was grown in MRS broth for 24 h (37 ºC, 5% CO2) and cell free solution was obtained by centrifugation for 15 min at 1500 ×g. and supernatant was neutralized by1N NaOH (1 mol/ lit) that adjust the pH to 6.5–7.0 to eliminate the effect of organic acids and the inhibitory effect of the hydrogen peroxide was eliminated by adding catalase according to Lieo, (1998). This neutralized supernatant was thereafter used in agar well diffusion and agar spot assays.

2.4. Antimicrobial activity of L. acidophilus on tested pathogens

Evaluating the antimicrobial activity of probiotic Lactobacillus against Cl. perfringens, E. coli and S. aureus were carried out with the following methods:

2.4.1. Time-Kill assay

Time-kill assay was conducted according to Prabhurajeshwar and Chandrakanth, (2019) by co-culturing each of the tested pathogens with L. acidophilus. Three ml of pathogen suspension (1.5×108 cfu/ml) was added to 3 ml of L. acidophilus (1.5×108 cfu/ml) in MRS broth and incubated at 37 ºC then serially diluted followed by culturing on TSC agar for Cl. perfringens, mannitol salt agar for S. aureus and Eosine methylene blue (EMB) for E. coli to determine the surviving cells of individual pathogens.

2.4.2. Agar well diffusion method

This technique was performed according to Weese et al. (2004). Two ml of 108 Cl. perfringens, E. coli and S. aureus were applied to soft MRS agar plates (containing 20 ml of medium). Plates were dried shortly at 37 ºC. Wells were made in each agar plate and 50-200 µL of L. acidophilus and its neutralized supernatant were added to the wells separately, while sterile peptone water was added into the control well. Plates were incubated anaerobically for 24 h at 37 □C. Each test was performed in duplicate. The formation of inhibition zone around the well was indicative of inhibitory activity of L. acidophilus. Inhibition zone was classified as: (-) non-visible inhibition, (+) 0.5-6 mm inhibition zone size, (++) 7-12 mm inhibition zone size, (+++) more than 12 mm inhibition zone size according to Perea Velez et al. (2007).

2.4.3. Agar spot test

This procedure was done as described by Anas et al. (2008) with some modifications. Briefly, in a- petri dish containing 10 ml of MRS agar, 3-5 µL of L. acidophilus inoculum and its neutralized supernatant were spotted separately onto one quadrant of the agar surface, followed by incubation at 37 □C for 24 h under anaerobic conditions. After incubation, 10 mL of BHI soft agar (BHI broth containing 0.7 % agar agar) containing 100 µL of E. coli or S. aureus inoculum and 10 mL of thioglycolate soft agar (thioglycolate broth containing 0.7 % agar agar) containing 100 µL of Cl. perfringens inoculum were overlaid onto the MRS agar separately. After solidification of the culture medium at room temperature (25–28 □C). The plates were incubated at 37 □C for 24 h under anaerobic conditions. Each test was performed in duplicate. The formation of a clear halo zone around the growth of the probiotics spot was indicative of antimicrobial activity. Inhibition zone was classified as described previously by Perea Velez et al. (2007).

2.4.4. Inhibition of gas production by Cl. perfringens

The ability of L. acidophilus to inhibit the growth of Cl. perfringens was evaluated by assessing the inhibition of gas production due to the fermentative action of the Cl. perfringens, as described by Golic et al. (2017) with some modifications. Briefly, this assay was performed by inoculating 1 µL of Cl. perfringens into 3 mL of soft RCM as lower layer (supplemented with 1.5 g/100 mL agar agar) that was homogenized by vortexing. Subsequently, 3 mL of soft MRS agar containing 0.7 g % agar was inoculated with 30 µL of L. acidophilus inoculum and its supernatant separately as upper layer that was homogenized by vortexing and immediately poured over the RCM agar layer. RCM agar with Cl. perfringens and MRS agar without inoculated L. acidophilus were used as negative controls. The tubes were incubated under anaerobic conditions at 37 □C for 24 h. This assay was performed in triplicate. Positive result for antimicrobial activity of L. acidophilus was characterized by the absence of gas production, which appear as absence of bubbles in the culture media, or medium breakage.

2.5. Aggregative abilities of L. acidophilus

Auto-aggregation and co-aggregation abilities of L. acidophilus were evaluated as follows:

2.5.1. Autoaggregation of L. acidophilus

Autoaggregation was conducted for L. acidophilus based on their deposition properties and for Cl. perfringens according to Collado et al. (2008) with slight modifications. L. acidophilus was grown for 18 to 24 h at 37□C on MRS broth and Cl. perfringens was grown for 18 to 24 h at 37 □C on BHI broth. The cells were harvested by centrifugation at 6000 g for 20 min, washed twice with phosphate buffer saline (pH 7.2) and the buffer was discarded and resuspended in phosphate buffered saline (PBS). Cell suspensions (4 ml) were mixed by vortexing for 10s and autoaggregation was determined during 4-24 h of incubation at room temperature. The results were evaluated visually, microscopically and by SEM.

2.5.2. Coaggregation of L. acidophilus with toxigenic Cl. perfringens type A

The coaggregation was performed to study the ability of L. acidophilus to coaggregate Cl. perfringens by two methods:

a.Tube method:

L. acidophilus and Cl. perfringens were separately cultured at 37 □C for 24 h in MRS and BHI medium, respectively. Bacterial suspensions were prepared as described in autoaggregation, with equal volume of cells of L. acidophilus and Cl. perfringens (1:1 v/v) mixed well by vortexing for 10 s and incubated at room temperature without agitation for 4-24 h. Tubes were observed macroscopically for visible clumps according to Collado et al. (2008). For microscopical visualization of bacterial coaggregations after Gram staining, glass slides were prepared with 5 µL of each suspension and scanning electron microscope (SEM) was evaluated according to Prabhurajeshwar and Chandrakanth, (2019)where it was done in faculty of medicine and faculty of science, Alexanderia university. Control assays were performed with individual bacteria (L. acidophilus and Cl. perfringens alone).

b.Plate method:

L. acidophilus and Cl. perfringens suspension were prepared as described previously in autoaggregation. According to Do Carmo et al. (2016), aliquots of 500 µL of L. acidophilus suspensions were mixed with 500 µL of Cl. perfringens suspension in 24-well plates and incubated at room temperature for 4-24 h under constant stirring (100 rpm) on an orbital shaker.

2.6. Viability assay of L. acidophilus with alpha toxin

According to Schoster et al. (2013), Guo et al. (2017), Prabhurajeshwar and Chandrakanth, (2019) with some modification, Alpha toxin of Cl. perfringens type A was used in co-culturing with L. acidophilus to investigate whether L. acidophilus colony forming unit (CFU) was affected by alpha toxin or not by using different concentrations of alpha toxin and different incubation time of co-culturing. Alpha toxin with 100 % concentration (80 minimum lethal dose (MLD)) and other concentrations were performed by dilution with peptonized saline (1g pepton + 8.5 g NaCl), 50 % (40 MLD) and 25 % (20 MLD). This assay was conducted by adding 3 ml L. acidophilus to 3 ml of each concentration of alpha toxin. The suspensions were incubated anaerobically for 4 h and 24 h at 37 □C. After incubation, the suspension was serially diluted and placed on MRS plates to determine the surviving cells of L. acidophilus. The suspension without alpha toxin was used as the control.

3. Results

3.1. Assessment of the potential antimicrobial activity of L. acidophilus against Cl. perfringens, E. coli and S. aureus by Time kill assay.

Time-kill assay revealed reduction in cell count of the three test pathogens in the presence of CFCS of L. acidophilus. The reduction in the viable colony count relative to the initial inoculum (1.5×108 cfu/ml) was 1.3×106, 1.2×10 and 4.4×105 cfu/ml for Cl. perfringens, E. coli and S. aureus, respectively, as shown in Fig 1. A, B and C and Fig 2.

3.2. Assessment of the potential antimicrobial activity of L. acidophilus against Cl. perfringens, E. coli and S. aureus by Agar well diffusion assay

To further asses the antibacterial activity of the selected L. acidophilus against Cl. perfringens, E. coli and S. aureus, agar well diffusion assay was performed. The zone of growth inhibition formed around the well containing L. acidophilus (≥ 5mm) appeared as a crescentic shape as shown in Fig. 3. 4 and 5.

3.3. Assessment of the potential antimicrobial activity of L. acidophilus against Cl. perfringens, E. coli and S. aureus by Agar spot test

To provide further insights into the antibacterial activity of L. acidophilus, agar spot test was performed. L. acidophilus inhibited the growth of Cl. perfringens, E. coli and S. aureus, which appeared as crescentic shape, and formation of halo zone around the coaggregation of L. acidophilus as shown in Fig. 6, 7. and 8.

3.4. Inhibition of gas production from Cl. perfringens by L. acidophilus

The ability of L. acidophilus to inhibit the growth of Cl. perfringens was evaluated by inhibition of gas production due to the fermentative action of Cl. perfringens. In case of presence of L. acidophilus, Cl. perfringens was not able to produce gas but in absence of L. acidophilus, Cl. perfringens was able to produce gas as shown in Fig. 9.

3.5. Autoaggregation and coaggregation of L. acidophilus with Cl. perfringens

Autoaggregation and coaggregation assay was examined visually, gram stain under light microscope and by SEM after incubation of L. acidophilus and Cl. perfringens together from 4 h to 24 h. It showed small clumps or aggregates that settled down in the bottom of the tube, indicating coaggregation as seen in Fig. 10 A, B, 11 and 12.

3.6. Viability Assay of L. acidophilus with Alpha toxin

when L. acidophilus incubated with different concentration of alpha toxin (25 %-50 %-100 %) with different incubation time (4 h-24 h), cfu of L. acidophilus were not remarkably affected as shown in Table (1) and Fig.13.

4. Discussion

Poultry industry has been affected by various impacts, including the emergence of variety of pathogens in addition to bacterial resistance, so there is an urgent need to find alternatives to control pathogens other than antibiotics. Probiotic is one of the alternative strategies to the use of antimicrobials in disease control. Therefore, we aimed to gain more insights into the antimicrobial activity of Lactobacillus against some pathogens (Cl. perfringens, E. coli and S. aureus) and investigate the co-aggregation potential with Cl. perfringens. Evaluation of the antimicrobial activity of L. acidophilus against these bacteria was performed by several analytical methods. Time-kill assay showed that L. acidophilus caused reduction in Cl. perfringens, E. coli and S. aureus from 1.5×108 cfu/ml to 1.3×106, 1.2×10 and 4.4×105 cfu/ml, respectively. These results agreed with Guo et al. (2017) who found that L. acidophilus greatly repressed the growth of Cl. perfringens after 20 h of incubation by 60 %. Ha Park et al. (2016) found that E. coli number decreased by 30% when treated with probiotics. Walencka et al. (2008) found that Lactobacilli are potent competitors to S. aureus. Prabhurajeshwar et al. (2017) recognized that Lactobacillus was an ideal potential in vitro antimicrobial probiotic against some pathogens. The use of Lactobacillus spp. on S. aureus and E. coli showed reduction in their cell counts and the killing effect was more effective with increasing the time of incubation. Several studies reported the effect of probiotics on the colonization of pathogenic bacteria as Mead (2000), who described that normal gut flora preparations showed efficacy against food borne pathogens such as E. coli and Clostridium spp. Maragkoudakis et al. (2006) and Charlier et al. (2008) reported that Lactobacillus are potentially promising because they generate bactericidal bioactive agents that are able to control the growth of pathogens as inhibition of Gram negative and positive pathogenic bacteria.

The results of agar well diffusion assay showed that both whole L. acidophilus and its neutralized CFCS have a strong inhibitory effect on Cl. perfringens, E. coli and S. aureus where clear halo zone of growth inhibition of the pathogens were formed and appeared as crescentic shape. Several studies using agar well diffusion assay as Gharaei-Fathabad and Eslamifar, (2011) who showed that Lactobacillus spp. have strong antibacterial activity against some clinically important pathogens such as E. coli and S. aureus. Osuntoki et al. (2008) used whole bacterium of different lactobacillus spp. and proved that they have antimicrobial action on different pathogens as E. coli, L. monocytogens and S. typhimurium. Lonkar et al. (2005) reported that L. acidophilus was active against E. coli. Mobarez et al. (2008) found that L. acidophilus exhibited antibacterial activities against S. aureus, Pseudomonas aeruginosa, Klebsiella pneumonia and Bacillus cereus. Aslim et al. (2005) showed that L. acidophilus has intermediate activity against S. aureus. On the other hand, Ayantola et al. (2016) found that supernatant of L. acidophilus did not affect E. coli, S. aureus and Shigella flexneri but S. typhi was affected.

The agar spot technique is considered another method to evaluate the antimicrobial activity of L. acidophilus. The results showed that L. acidophilus inhibited the growth of Cl. perfringens, E. coli and S. aureus and appeared as crescentic shape with formation of clear halo zone of growth inhibition of pathogens formed around the spot of both L. acidophilus inoculum and its neutralized CFCS and appeared as crescentic shape. Several studies agree with these results as Anas et al. (2008) who performed this procedure between lactobacilli and S. aureus and showed the formation of a clear halo around growth of the probiotics. Gharaei-Fathabad and Eslamifar, (2011) used whole bacterium of Lactobacillus spp. which had a strong antibacterial activity against some clinically important pathogens such as E. coli, S. aureus, S. typhi and citrobacter spp. Mami et al. (2012) investigated the antagonistic activity of both whole Lactobacillus and its CFCS against variety of microorganisms as S. aureus, E. coli and Bacillus spp., when measuring the diameter of the inhibition zones, it showed that the Gram-positive bacteria (S. aureus) were more sensitive to the inhibiting substances produced by the lactobacilli compared to the Gram-negative bacterium (E. coli). Using both whole bacterium and cell free culture supernatant (CFCS) of L. acidophilus in previous assays gave the same antibacterial activity, suggesting that antimicrobial activity of L. acidophilus was not related to the acidity only but also possibly to other antibacterial substance(s) could have been produced by L. acidophilus in the CFCS. This agrees with Coconnier et al. (1997), Pascual et al. (2008) and Prabhurajeshwar et al. (2017). On the contrary, Banina et al. (1998) explained the inhibition of gas production to be due to lactic acid production by L. acidophilus rather than hydrogen peroxide or bacteriocin. The antibacterial test results when compared with the two methods (agar overlay and agar-well diffusion) employed in the current study, was in accordance with the results reported by Cadirci and Citak (2005) who inspected antagonism of Lactobacilli against Gram-negative bacteria using the above two methods and found that the spot method (agar overlay method) was the effective one in the evaluation of the inhibitory activity, However, Rahimifard and Naseri (2016) showed that the well diffusion method was the best to evaluate antagonism than the other two methods (disk diffusion and agar spot technique) employed. Halder et al. (2017) reported that Lactobacilli had excellent antibacterial activity in agar-well as well as agar overlay methods. The variation in antibacterial activities as depicted by different studies might be due to the number of CFU of the lactobacilli used (in spot method) and/or the amount of culture used (in agar well diffusion) as well as the antibacterial product activity possessed in it as has been reported by Iyapparaj et al. (2013) and Shehata et al. (2016). Cl. perfringens is capable of gas production due to its fermentative action, this ability can be inhibited by L. acidophilus which has a strong antimicrobial activity against Cl. perfringens, both whole L. acidophilus and its CFCS gave the same antimicrobial effect. Banina et al. (1998) showed that whole bacterium of L. acidophilus exhibited an inhibitory effect on the growth of Clostridia by inhibition of its gas production. Golic et al. (2017) found that five Lactobacillus strains and their CFCS had inhibitory activity on gas production by Clostridium butyricum. Coman et al. (2014) and Monteiro et al. (2019) found that Lactobacillus spp. present different levels of antimicrobial efficacy against Cl.

butyricum, Cl. difficile, and Cl. perfringens by inhibition of their gas production.

Auto-aggregation and coaggregation are of extensive importance in several ecological niches. coaggregation may promote biofilm development, changes in biofilm architecture, and altered species composition of biofilm (Kolenbrander et al. (2006), Hojo et al. (2009)). Autoaggregation and coaggregation of L. acidophilus were examined by using broth-grown cells of L. acidophilus, suspended in their own culture fluid then resuspended in PBS because the method of culture has been recognized as a factor that may affect bacterial aggregation. This agreed with Kos et al. (2003) who found that there was a strong autoaggregating phenotype of L. acidophilus that was not lost after washing and suspending of the cells in PBS and better growth of the bacterium on MRS broth than on MRS agar. The observed autoaggregation could be related to cell surface component because it was not lost after washing and suspending of the cells in PBS. The results of autoaggregation and co-aggregation assay of L. acidophilus and Cl. perfringens were examined visually showing large clumps or aggregates that settled down in the bottom of the tube, microscopically by gram stain under light microscope and scanning electron microscope (SEM), showing L. acidophilus as coccobacilli in shape, the length of the cells varied from 2.02 µm to 5.49 µm and the diameter ranged from 0.50 µm to 0.59 µm. The shape and the range of the measured dimension were in good agreement with the results of Ray et al. (2001) and Pyar et al. (2014). Cl. perfringens appeared as large rectangular bacilli with rounded or truncated ends, pleomorphic with straight or curved rods, size is about 3-8 µm X 0.4-1.2 µm, capsulated, non-motile and non-flagellated pathogenic bacteria and containing spores with central or sub-terminal spores but spores are rare as published by Monteiro et al. (2019). Also, scanning electron microscope (SEM) explained coaggregation assay between L. acidophilus and Cl. perfringens all happened in relation to time that were highest at the 4 h of incubation time and lasted for 24h. These results agree with Collado et al. (2008) and Prabhurajeshwar et al. (2017) who used three probiotic Lactobacillus spp. and seven different test pathogens and used bacterial suspension (108 cfu/ml) for aggregation assay. Katharios-Lanwermeyer et al. (2014) considered the visual coaggregation assay to be more rapid, less technically complex and generates results that are often more reproducible than other techniques to study coaggregation. On the other hand, Raouf et al. (2013) found that it is difficult to notice small changes in cell morphologies of bacteria under the light microscope, so SEM was used in the present investigation to review the changes or damage in cell morphology of the populations by the effect of coaagregation with lactobacillus that what seen in this study.

Alpha-toxin is considered the key risk factor for inducing necrotic enteritis. Once Cl. perfringens population reaches a certain density (>104 CFU/g), toxin production is triggered, which induces Cl. perfringens infection (Sawires et al. (2006), Logue et al. (2013)). Guo et al. (2017) found that L. acidophilus decreased the α-toxin production by Cl. perfringens without influencing its biomass, and even degraded the established α-toxin. This study confirmed the importance of L. acidophilus inhibitory activity on alpha toxin of Cl. Perfringens type A as it was important to investigate if alpha toxin have adverse effect on L. acidophilus in vitro, this was assessed by coculturing of L. acidophilus and alpha toxin. We found that L. acidophilus cell number was not remarkably affected by incubation with different concentrations of alpha toxin and different incubation time. This result agrees with previous results as L. acidophilus have antimicrobial activity against Cl. perfringens and not affected by its alpha toxin.

In conclusion, this study proved the antimicrobial effect of L. acidophilus on Cl. perfringens type A producing alpha toxin, E. coli and S. aureus. L. acidophilus has marked aggregative abilities. Moreover, there was no inhibitory effect of alpha toxin on L. acidophilus. Therefore, L. acidophilus could play an important role in resisting NE in broilers farms, however further in vivo assessment is required to study the host response and the relationship between L. acidophilus and alpha toxin of Cl. perfringens.

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Conflict of interests

The authors have not declared any conflict of interests.

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Table (1): Antimicrobial activity between alpha toxin and L. acidophilus

Toxin conc (%)	L. acidophilus count	
	4 h Incubation	24 h Incubation
25	0.6×108	3.5×108
50	1.6×107	8×107
100	1.1×107	6×107

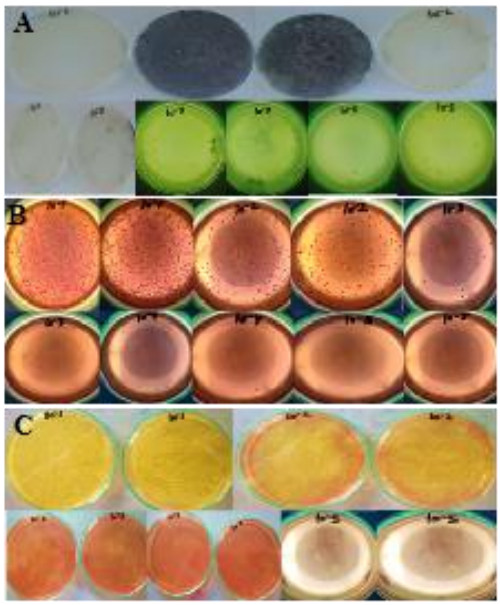

Fig. 1. Time-kill assay of L. acidophilus on: (A) Cl. perfringens showing reduction in cfu of Cl. perfringens. (B) E. coli showing reduction in cfu of E. coli from 1.5×108 cfu/ml to 1.2×104. Duplication each dilution according to ISO 17025. (C) S. aureus showing reduction in cfu of S. aureus from 1.5×108 cfu/ml to 4.4×105 . Duplication each dilution according to ISO 17025.

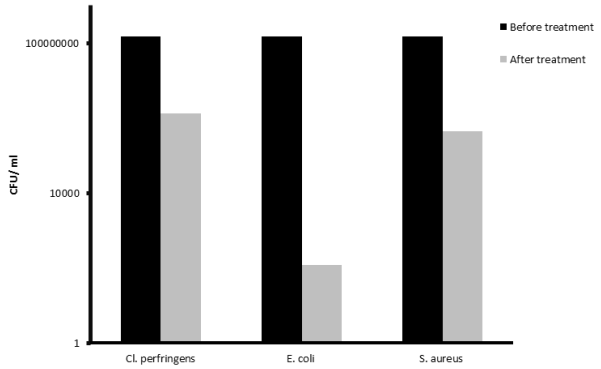

Fig. 2. Time kill assay of L. acidophilus (CFCS) against Cl. perfringens, E. coli and S. aureus showing reduction in their CFU.

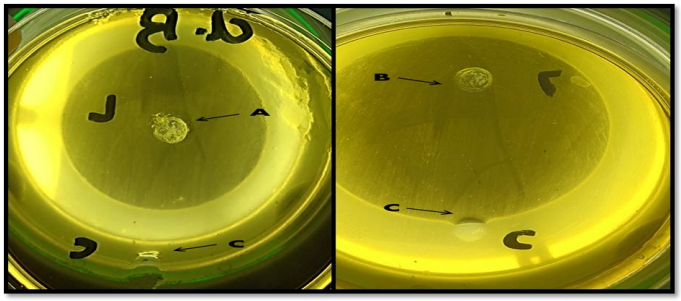

Fig. 3. Antimicrobial effect of L. acidophilus on Cl. perfringens growth by agar well diffusion method showing (A) Whole bacterium L. acidophilus forming clear halo zone of growth inhibition of Cl. perfringens (5 mm) appearing as a crescentic shape. (B) Neutralized L. acidophilus supernatant forming clear halo zone of growth inhibition of Cl. perfringens (5 mm) appearing as a crescentic shape. (C) Growth of Cl. perfringens around control well.

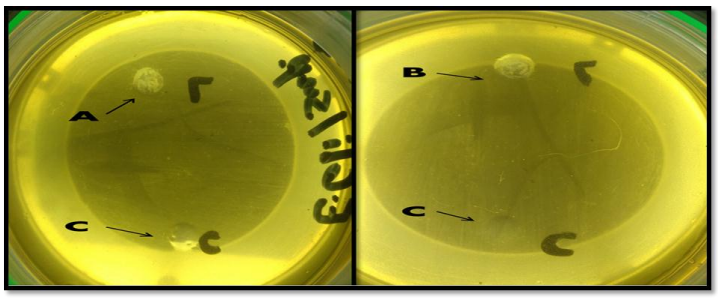

Fig. 4. Antimicrobial effect of L. acidophilus on E. coli growth by agar well diffusion method showed (A) Whole bacterium L. acidophilus forming clear halo zone of growth inhibition of E. coli (5 mm) appearing as a crescentic shape. (B) Neutralized L. acidophilus supernatant forming clear halo zone of growth inhibition of E. coli (5 mm) appearing as a crescentic shape. (C) Growth of E. coli around control well.

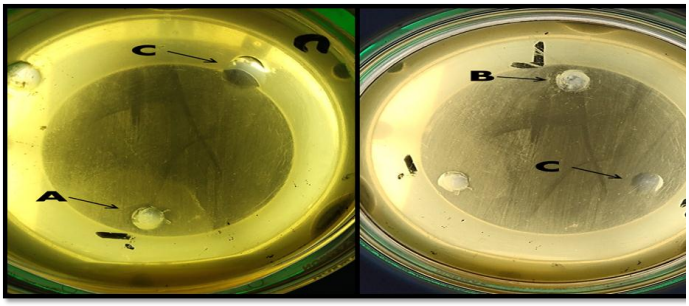

Fig. 5. Antimicrobial effect of L. acidophilus on S. aureus growth by agar well diffusion method showed (A) Whole bacterium L. acidophilus forming clear halo zone of growth inhibition of S. aureus (5 mm) appearing as a crescentic shape. (B) Neutralized L. acidophilus supernatant forming clear halo zone of growth inhibition of S. aureus (5 mm) appearing as a crescentic shape. (C) Growth of S. aureus around control well.

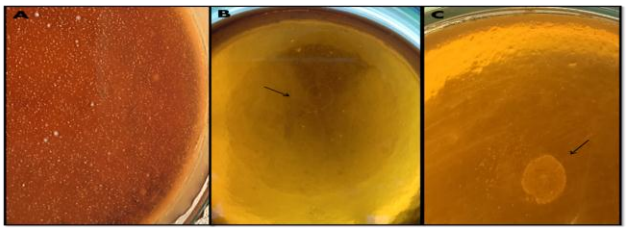

Fig. 6. Antimicrobial effect of L. acidophilus on Cl. perfringens growth by agar spot technique: (A) Growth of Cl. perfringens on MRS medium without L. acidophilus (control). (B) Whole bacterium L. acidophilus spot forming clear halo zone of growth inhibition of Cl. perfringens (≥ 6mm) around the aggregation of cells around the spot, appearing as a crescentic shape. (C) Neutralized CFCS of L. acidophilus spot forming clear halo zone of growth inhibition of Cl. perfringens (≥ 6mm) around the aggregation of cells around the spot, appearing as a crescentic shape.

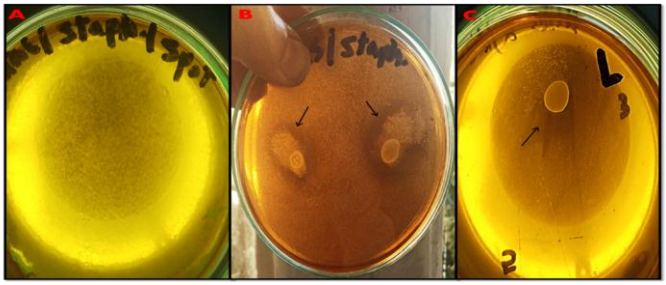

Fig. 7. Antagonistic effect of L. acidophilus on E. coli growth by agar spot technique showed (A) Growth of E. coli on MRS medium without L. acidophilus (control). (B) Whole bacterium L. acidophilus spot forming clear halo zone of growth inhibition of E. coli (≥ 6mm) around the aggregation of cells around the spot, appearing as a crescentic shape. (C) Neutralized CFCS of L. acidophilus spot forming clear halo zone of growth inhibition of E. coli (≥ 6mm) around the aggregation of cells around the spot, appearing as a crescentic shape.

Fig. 8. Antagonistic effect of L. acidophilus on S. aureus growth by agar spot technique showed (A) Growth of S. aureus on MRS medium without L. acidophilus (control). (B) Whole bacterium L. acidophilus spot forming clear halo zone of growth inhibition of S. aureus (≥ 6mm) around the aggregation of cells around the spot, appearing as a crescentic shape. (C) Neutralized CFCS of L. acidophilus spot forming clear halo zone of growth inhibition of S. aureus (≥ 6mm) around the aggregation of cells around the spot, appearing as a crescentic shape.

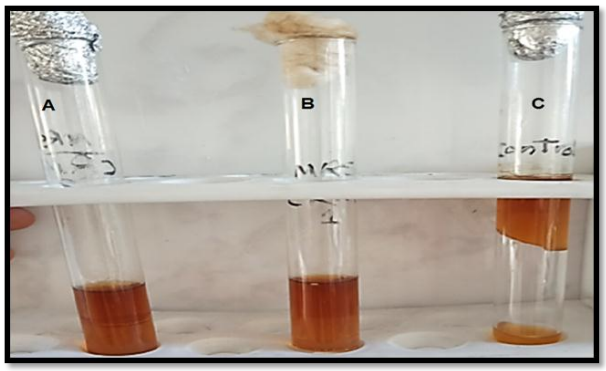

Fig. 9. Effect of L. acidophilus on Cl. perfringens gas production (A) Using whole bacterium L. acidophilus showing inhibition of gas production of Cl. perfringens. (B) Using CFCS L. acidophilus showing inhibition of gas production of Cl. perfringens. (C) control tube showing gas production of Cl. perfringens in absence of L. acidophilus.

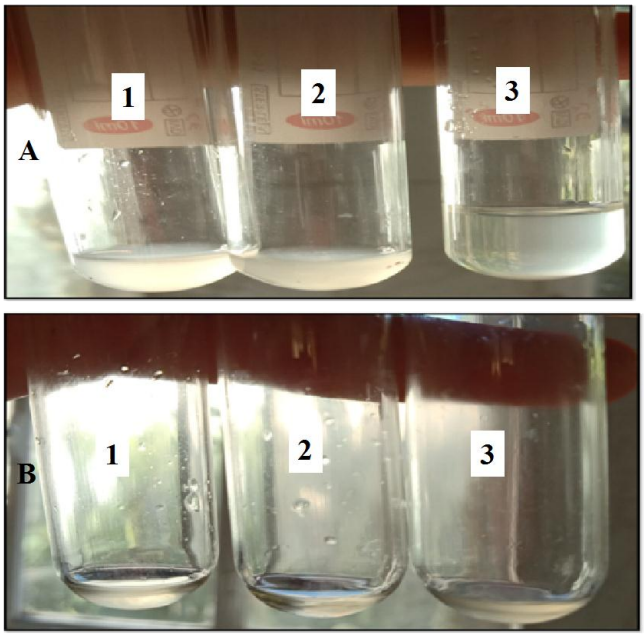

Fig. 10. Visual examination of autoaggregation and coaggregation:
(A) within 4hr: (1) No coaggregation between L. acidophilus and Cl. perfringens showing whole turbidity (2) No autoaggregation of L.

acidophilus showing whole turbidity (3) No autoaggregation of Cl. perfringens showing whole turbidity.
(B) within 24hr: (1) Coaggregation showing small clumps or aggregates settle down in the bottom of the tube due to incubation of L. acidophilus and Cl. perfringens for 4h - 24h at room temperature. (2) Autoaggregation showing small clumps or aggregates settle down in the bottom of the tube due to incubation of L. acidophilus only for 4h - 24h at room temperature. (3) No autoaggregation showing whole turbidity due to Cl. perfringens incubated alone for 4h - 24h at room temperature.

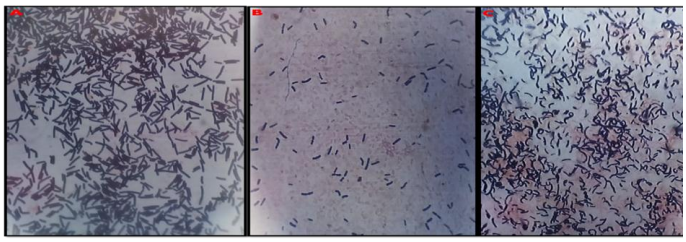

Fig. 11. Gram stain of autoaggregated and coaggregated bacteria: (A) L. acidophilus showing autoaggregation. (B) Cl. perfringens showing no autoaggregation (C) Coaggregation L. acidophilus and Cl. perfringens.

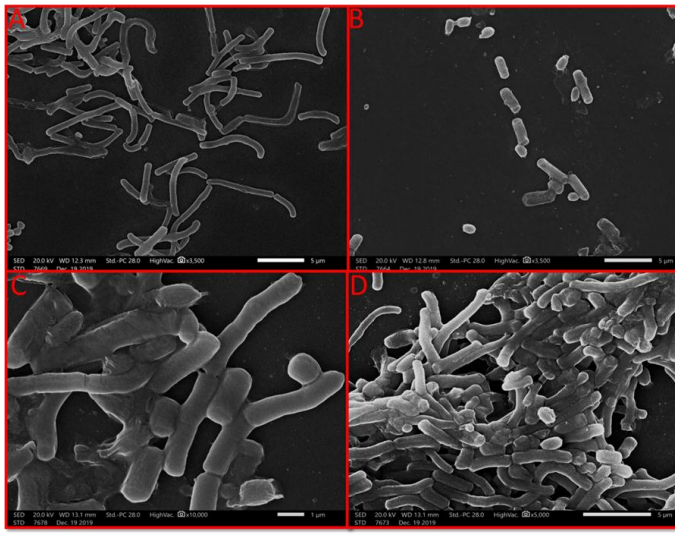

Fig. 12. Scanning electron microscope of (A) L. acidophilus showing autoaggregation (magnification 10,000x). (B) Cl. perfringens showing no autoaggregation and a regular outlined cell wall (magnification 3,500x) (C) Coaggregation L. acidophilus and Cl. perfringens which appeared as disrupted cell wall (magnification 10,000x). (C) Coaggregation L. acidophilus and Cl. perfringens which appeared as disrupted cell wall (magnification 3,500x).

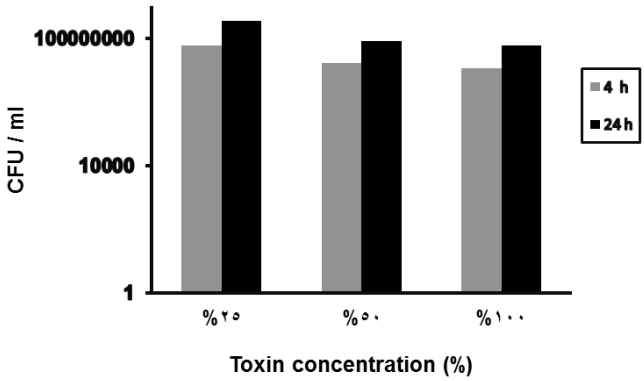

Fig. 13. Effect of alpha toxin on L. acidophilus showing that when L. acidophilus incubated with different concentration of alpha toxin (25%-50%-100%) with different incubation time (4hr-24hr), cfu of L. acidophilus not remarkable affected.