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Comparison of *in vitro* and *in situ* antagonism assays as tools for the selection of bio-preservative lactic acid bacteria (LAB) in poultry meat

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ABSTRACT

The study compared 3 antagonism assays, *In vitro* in broth culture (TSBYE) and *In situ* in sterile and raw chicken meat, as tools for LABs selection to bio-preserve poultry against *Pseudomonas* spoilage. To do this, 4 LABs (≈8 log CFU/mL or cm^2) were inoculated with one *P. fragi* (≈3 log CFU/mL or cm^2) on TSBYE or irradiated poultry, or alone on raw chicken meat (*Pseudomonas* spp. ≈3 log CFU/cm^2). Treatments were incubated at 8 °C along time (4–6 days). Bacterial counts were performed on selective agar.

In vitro assay was technically easy to perform, but antagonistic LABs abilities observed, were not always consistent with those evidenced *In situ*. Assay on irradiated meat demonstrated a good predictive potential for LABs selection, but not to discharge them. The main disadvantage of both assays was that sensitivity of *P. fragi* not necessarily represented the sensitivity shown by *Pseudomonas* spp. within poultry microbiota. In contrast, trials on raw poultry showed the real bio-preservative capacity of LAB against *Pseudomonas* spp., but variability within replicates was high.

In conclusion, assay on fresh poultry must always be performed to select bio-preservative LABs, while other experiments are useful for reducing the number of LABs to be assayed.

1. Introduction

Poultry meat has become the world's most consumed meat protein in 2019 (OECD, 2019), but is highly perishable commodity. Early spoilage of meat can lead to food waste and thereby economic losses as well as the loss of consumer confidence (Bruckner, Albrecht, Petersen, & Kreyenschmidt, 2012).

Poultry spoilage is mainly due to the growth of psychrotrophs spoilage microorganisms on the meat surface, even at -4 °C (Bailey, Lyon, Lyon, & Windham, 2000). The spoilage microbiota varied according the producer, type of chicken meat (i.e., whole meat and chicken breast) and storage temperatures (Lee, Kwon, Heo, Kim, & Kim, 2017; Morales, Aguirre, Troncoso, & Figueroa, 2016a; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Regardless, multiple authors report *Pseudomonas* spp., as the predominant spoilage microorganisms in aerobic poultry meat; Morales et al. (2016a) established that the dominant specie was *P. fragi*, followed by *P. fluorescens* and *P. lundensis*, while other authors isolated mainly *P. weihenstephanensis* and *P. psychrophila* (Lee et al., 2017) or *P. putida* (Bruckner et al., 2012) at the end of shelf life of poultry packed aerobically.

In order to avoid *Pseudomonas* spoilage and thus extend product shelf life, different treatments, including modified atmosphere or vacuum packaging and chemical preservatives, are used (Narasimha & Sachindra, 2002; EFSA, 2008; FAO/WHO, 2008). But actually, consumers encourage the food industry to limit the use of plastic or chemical compounds and develop natural methods for preservation (Roman, 2017).

Bio-preservation meets with the demand of consumers. It is based on microbial antagonism, where nonpathogenic strains (or their metabolites) antagonize foodborne pathogens or spoiling bacteria (Da Costa et al., 2019). Lactic acid bacteria (LAB) is an heterogeneous microbial group most used as protective cultures in foods (Gaggia, Gioia, Baffoni, & Biavati, 2011). Specifically, some *Lactobacillus* spp. Are commonly inoculated in fermented or cooked meat byproducts and vacuum-packed meat to extend their shelf life and enhance food safety (Casaburi, Di Martino, Ferranti, Picariello, & Villani, 2016; Goodarzi, Hovhannisyan, & Barseghyan, 2016; Katikou, Ambrosiadis, Georgantelis, Koidis, & Georgakis, 2005; Vermeiren, Devlieghere, & Debevere, 2006), but is less used on aerobic fresh meat and poultry, where the aim was to bio-control *Listeria monocytogenes*, *Salmonella enteritidis* or *E. coli* O157H7 (Braïek et al., 2019; Da Costa et al., 2018). BAL antagonistic ability is due to the production of several

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Table 1
Resume of protective strains used in the study.

Specie	Identification	Source	Grown conditions	Reference
L. sakei	L.A	Spoiled chicken meat	Aerobiosis, 30 °C	Morales (2019).
L. sakei CECT 4808	L.B	Spanish Type Culture Collection (CECT)	Aerobiosis, 30 °C	Katikou et al. (2005)
Lactobacillus spp.	L.C	Cecal poultry content	Anaerobiosis, 37 °C	Melgarejo (2011).
L. rhamnosus	L.D	Human stools	Anaerobiosis, 37 °C	Gotteland et al. (2014)

antimicrobial compounds, such bacteriocins, organic acids, diacetyl, H_2O_2 and/or reuterin (Collins, Cotter, Hill, & Ross, 2010), and their production rate depends on nutrients availability in culture medium (Aasen, Møretrø, Katla, Axelsson, & Storrø, 2000).

Bio-preservative BAL should be selected taking into consideration its effectiveness in the particular food product and its range of microbial activity, because sometimes is limited to a specific species (Silva et al., 2018). Then is vital to known the pro and cons of antagonisms assays and perform an optimal protocol to create commercial bio-preservatives to poultry industry. Then the aim of this study was to compare the usefulness of *in vitro* antagonism assay in broth cultures and *in situ* tests using irradiated and fresh poultry, both packed in aerobiosis, as tools for the selection of potential lactic protective cultures that delay the grown of spoilage *Pseudomonas* spp.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Protective cultures: 4 *Lactobacillus* strains were included in this study. Source, growth conditions and species are shown in Table 1. *L. sakei* (LA), *Lactobacillus* spp. (LC) and *L. rhamnosus* (LD) were previously identified by 16sRNA sequence (Gotteland et al., 2014; Data not shown). All strains were grown in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, UK), during 24–48 h prior tests.

Challenged strain*:* Poultry spoilage *P. fragi*, representing the prevalent *Pseudomonas* isolated from spoilage skinless marinated poultry breast fillets at 4 °C (Morales et al., 2016a), was inoculated in co-cultures antagonisms assays. The strain was grown in CFC agar (Pseudomonas agar base (Oxoid, UK) plus a selective supplement made with Cetrimide, Fusidin and Cephalosporin (CFC; Oxoid, UK)) or Trypticase Soy Broth (BD, USA) with 0.6% Yeast Extract (BD, USA) (TSBYE) during 24–48 h prior tests.

2.2. In vitro competitive inhibition in broth culture at 8 °C

P. Fragi was challenged by lactic treatments detailed in Table 2 according Doyle and Zhao (2009) with a few modifications. Briefly, 80 mL TSBYE (pH 5.7–5.9) was inoculated with 10 mL *P. fragi* ($\approx 10^4$ CFU/mL) adjusted with TSBYE and 10 mL protective culture ($\approx 10^9$ CFU/mL) in modified MRS broth (without Gram negative antagonisms ingredients; Peptone 10 g/L, Beef extract 10 g/L, Yeast extract 5 g/L, Dextrose 20 g/L, Potassium phosphate 2 g/L, Magnesium sulphate 0,1 g/L, Manganese sulphate 0,05 g/L, Tween® 80 1 mL/L; MRSm) or MRSm broth as negative control. Then cultures were allocated in 8 mL tubes and incubated at 8 ± 2 °C in aerobiosis without stirring by 6 days. Bacterial counts were performed on 0, 2, 4 & 6 days. Additionally pH of each treatment was recorded with pH paper indicator (Merck, Germany).

Table 2
In vitro antagonisms treatments inoculated in TSBYE incubated in 8 ± 2 °C.

Treatment	Lactic protective culture		Challenged spoilage bacteria	
T0	–		*Ps. fragi*	10^3 CFU/mL
T1	L.A	**10^8** CFU/mL		
T2	L.B			
T3	L.C			
T4	L.D			

2.3. In situ competitive inhibition in irradiated chicken meat at 8 °C

Poultry skinless unmarinated breast fillets were purchased at local supermarket, cutted manually (3 × 3x1 cm), frozen at −18 °C and sterilized by gamma ionization at the Chilean Commission of Nuclear Energy (CCHEN) using a dose of 15 kGy by 7 h.

Then 60 pieces were randomly assigned into one of each treatment with positive bio-preservative potential in TSBYE (previous experiment) described in Table 3. Only one meat surface (9 cm^2) was inoculated with both bacterial cultured previously washed, re-suspended and adjusted in NaCl 0.85% sterile solution (Merck, Germany). All treatments were incubated at 8 ± 2 °C in aerobiosis without stirring by 4 days. The growth and survival of both protective culture and spoilage bacteria was monitored every days with microbiological analysis.

2.4. In situ protective cultures application in fresh chicken meat at 8 °C

Poultry samples were randomly purchased in trays of skinless unmarinated poultry breast fillets from one producer. The fillets were obtained on the first day on sale at a local supermarket from a shelf at 4 °C. The sample was immediately transported under refrigeration at 4 °C to the Lab where meat was cut, within 45 min.

Poultry cubes ($\approx$1 cm^3; n = 400) were randomly allocated into one of each treatment (T1 & 2) with some positive *in situ* bio-preservative potential in sterile meat (previous experiment) and into one treatment without protective potential *in vitro* in TSBYE (T3) (Table 4).

Experimental inoculation was performed in sterile bags (Fig. 1A). 100 meat cubes were contaminated with 10 mL of protective culture ($\approx 10^{11}$ CFU/mL) with a glass pipette and manually homogenized (Fig. 1a). Each treatment were divided in sterile petri dishes, each with 10 cuts (Fig. 1b). All meat samples were kept under domestic refrigeration conditions (8 ± 2 °C) and the growth and survival of both protective cultures and *Pseudomonas* spp. were monitored at 0, 2, 4 and 6 days. Additionally, at the end of each assay, the effect of treatments were compared under UV light and photographed.

2.5. Microbiological analysis

Bacterial counts, protective cultures and *P. Fragi* or *Pseudomonas* spp., were performed using standard serial dilution method with peptone water 0.1% (Merck, Germany). *P. fragi* or *Pseudomonas* spp. were enumerated on selective CFC agar at 22 °C in aerobiosis for 48 h, while

Table 3
In situ antagonisms treatments inoculated in sterile chicken meat incubated in 8 ± 2 °C.

Treatment	Lacticprotective culture		Challenged spoilage bacteria	
T0	NaCl 0.85%		*Ps. fragi*	10^3UFC/cm^2
T1	L.A	**10^8** CFU/cm^2		
T2	L.B			
T3	L.D			

Table 4
In situ antagonisms treatments inoculated in fresh chicken meat.

Treatment	Lactic protective culture	
T0	NaCl 0,85%	
T1	L.A	10^8 UFC/cm^2
T2	L.B	
T3	L.C	

protective cultures counts were monitored on selective MRS medium at 30 °C in aerobiosis (L.A & L.B) or at 37 °C in anaerobiosis (L.C & L.D) for 48 h.

2.6. Statistical analysis

Bacterial counts were reported as Log_{10} colony forming units per volume or surface units (log CFU/mL o cm^2) and expressed as mean.

To compare the effect of the LAB to bio-control *P. fragi* or *Pseudomonas* spp. in each experiment, mathematical models were performed for each trial and analyzed by general and mixed linear model using Infostat® software (version 2017p, Argentine), modeling the correlation between different times and the variance if necessary, followed by Fisher's LSD post hoc test, with Bonferroni p-value correction procedure. Statistically significant differences were reported at $p < 0.05$.

3. Results and discution

All 3 assays, *In vitro* competitive inhibition in TSBYE, *In situ* competitive inhibition in irradiated or fresh chicken meat, evidenced an effect ($p < 0.01$) of the treatments on *P. fragi* or *Pseudomonas* spp. counts, depending on the interaction between the treatment and the time evaluated (Figs. 2–4), however, the magnitude of the antagonism and the utility of each test showed differences as discussed below.

3.1. In vitro competitive inhibition in broth cultures at 8 °C

The greatest *in vitro* bio-preservative potential in TSBYE at 8 °C was evidenced with both *L. sakei* (T1 and T2) and less with *L. rhamnosus* (T4); at day 6 of assay, LAB inhibition rate were 99.99999% (T1 and T2) and 99.9% (T4) (Fig. 2).

These results demonstrate that bio-control activity depends on LAB growth abilities at low temperature; *L. sakei* is adapted to grown at cold temperatures and not *L. rhamnosus* (Chaillou et al., 2005; Hammes & Hertel, 2007). This is in agreement with other studies such Goodarzi et al. (2016) and partially with Amézquita and Brashears (2002), who reported that the bio-preserving potential of LABs occurs even when they do not grow, once they are added to the food in high concentrations ($> 10^7$ CFU/g).

The antagonism evidenced by both *L. sakei* and *L. rhamnosus* can be explained, in part, by their capacity to produce organic acids and reduce the pH of the medium (Collins et al., 2010), both reaching a pH 4.4 on day 4. In contrast, *Lactobacillus* spp. (T3) without bio-control capacity, lowered ph until 5.0 the same day. The higher antagonist potential of *L sakei* compared to *L. rhamnosus* may be due to the concomitant production of diacetyl, acetoin, ethanol, hydrogen peroxide and/or bacteriocins that act synergistically with organics acids to exert antagonism (Chaillou et al., 2005).

In vitro competitive inhibition in TSBYE is an ideal assay to compare the bio-preservative potential of several lactic bacteria at the same time, and to pre-select potential protective cultures, due to its simplicity and technical easy management. However, the results cannot be directly extrapolated to bacterial kinetic behavior and bio-preservative activity in poultry, mainly due to:

- Challenge a finite number of spoilage bacteria strains. In general, *in vitro* assays only confront the protective culture to one or a few strains of the species to be challenged (Santini et al., 2010; Zhao, Doyle, & Zhao, 2004); in this study *P. fragi* represents the whole *Pseudomonas* spp., while Morales et al. (2016a) revealed a high level of variability between and within dominant *Pseudomonas* species isolated from poultry. Selecting representative strain of a species is simple, but increase in uncertainty and does not ensure the representativeness of the sensitivity of the specie. Strain variability should be included in *in vitro* challenge assays to optimize the bio-preservative cultures selection (Lianou & Koutsoumanis, 2011).
- Culture broth medium has different component and states of aggregation than poultry meat (Aasen et al., 2000; Moretro, Aasen, Storro, & Axelsson, 2000). Therefore, the production and diffusion rate of the antimicrobial metabolites released by the LABs will be different in both media (Aasen et al., 2000; Moretro et al., 2000; Shao et al., 2017).

3.2. In situ competitive inhibition in irradiated chicken meat at 8 °C

In situ bio-preservative potential in irradiated chicken meat at 8 °C was evidenced only with *L. sakei* L.A (T1). On day 2 and 3 the *P. fragi* counts were 4.39 and 6.45 $\log_{10}$ CFU/cm^2 for *L. sakei* L.A compared with 5.16 and 7.17 $\log_{10}$ CFU/cm^2 for the control, respectively; *L. sakei* L.A grew ≈ 1 $\log_{10}$ CFU/cm^2 by the first day of assay and then remained constant (Fig. 3).

Statistical analysis demonstrated that *L sakei* L.B (T2) also antagonized with *P. fragi,* however, the difference between this treatment and

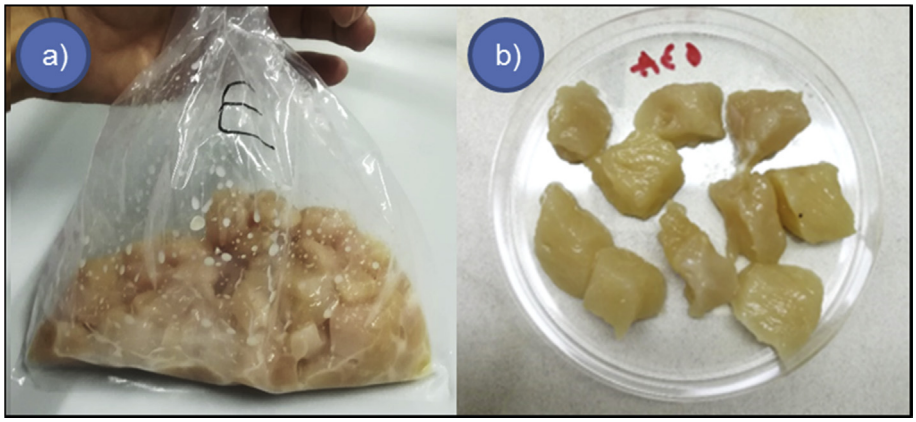

Fig. 1. Photos preparation of *in situ* protective cultures application in fresh chicken meat at 8 °C. a) Experimental inoculation of 100 cubes of chicken meat with 10 mL of protective culture. b) Inoculated 10 meat cubes in petri dishes incubated at 8 °C.
*Bare-hand was cropped.

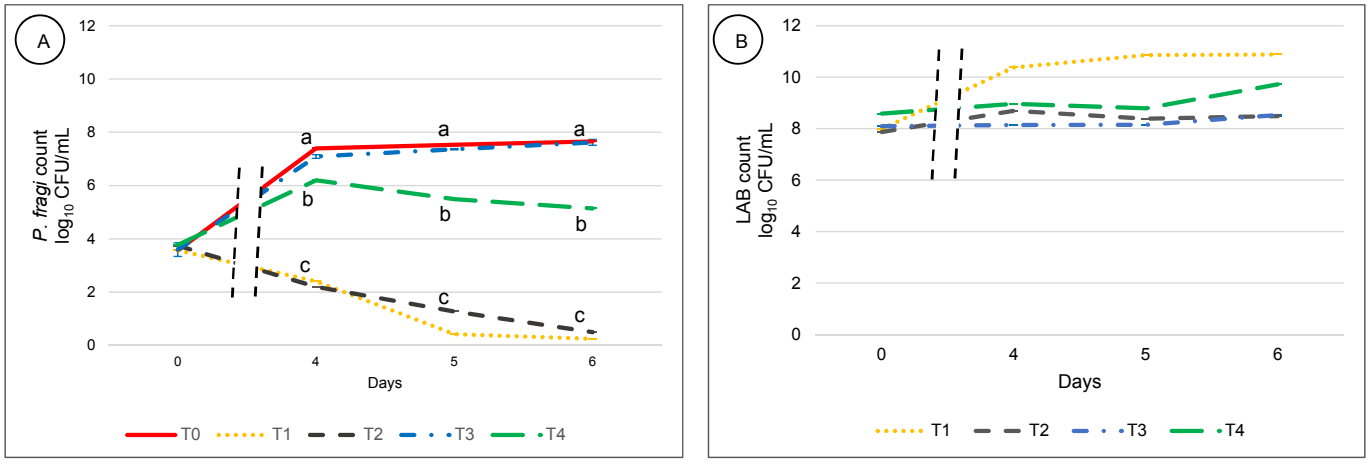

Fig. 2. *P. Fragi* (A) and LAB (B) counts (mean ± SD Log_{10} CFU/mL) in *In vitro* competitive inhibition assay in TSBYE at 8 °C during 6-day storage period. *T0 (Red) Control treatment, ≈3 log_{10} CFU/mL *P. fragi* without LAB (inoculated with NaCl 0,85%); T1 (Yellow), ≈3 log_{10} CFU/mL *P. fragi* inoculated with ≈8 log_{10} CFU/mL *L. sakei* L.A; T2 (Gray), ≈3 log_{10} CFU/mL *P. fragi* inoculated with ≈8 log_{10} CFU/mL *L. sakei* CECT 4808 L.B; T3 (Blue), ≈3 log_{10} CFU/mL *P. fragi* inoculated with ≈8 log_{10} CFU/mL *Lactobacillus* spp. L.C; T4 (Green), ≈3 log_{10} CFU/mL *P. fragi* inoculated with ≈8 log_{10} CFU/mL *L. rhamnosus* (L.D). TSBYE: Trypticase Soy Broth with 0.6% Yeast Extract. Different letters indicate significant statistical differences between treatments by day ($p < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control (T0) is less than 0.5 log, therefore, it has no real microbiological or technical sense (Fig. 3). This was not expected when this strain antagonized with *Pseudomonas* spp. in red meat (Katikou et al., 2005).

The low antagonistic abilities of lactic strains against *P. fragi* demonstrated in this assay, using irradiated meat, contradict with those evidenced in TSBYE (Fig. 2). Below are possible reasons that could explain this fact.

P. fragi, the challenged spoilage strain, was isolated from poultry meat, therefore, it is a strain adapted to growth in this environment (Morales et al., 2016a). Moreover, irradiated meat was sterile (unpublished data), so *P. fragi* had no concomitant antagonism due to other microbial competence beyond inoculated lactic strains. *P. fragi* was intentionally selected, because of its mayor sensitivity against 27 strains of *Lactobacillus* spp. in double agar layer antagonism assay (Morales, Troncoso, & Figueroa, 2016b).

LABs produce and spread different amounts of antibacterial metabolites depending on the medium where they are incubated (nutrients, state of aggregation and others) (Aasen et al., 2000; Moretro et al., 2000).

The concentration of free glucose in culture medium has been identified as a critical factor for fermentation and organic acids production by LABs. The greater availability of carbohydrate in the medium will determine greater amounts of organic acids and faster decrease in pH, achieving the antagonistic effect (Lücke, 1994; Shiraia et al., 2001). TSBYE contains dextrose (D-glucose) (BD, 2008), therefore, it is easily metabolizable by bacteria, whereas meat contains small amounts of glucose, mainly stored as glycogen within muscle cells, being little available and difficult to metabolize (Lawrie, 1998; Lücke, 1994).

Aasen et al. (2000) showed that bacteriocin production of *L. sakei* increases proportional to the concentration of yeast extract and tryptone contained in medium. Both factors are found in TSBYE, not in poultry meat.

The different state of aggregation of the matrices used in assays (TSBYE-liquid v/s meat-solid) affects the diffusion rate of both microorganisms and antibacterial substances released to the medium. In liquid, the dispersion of bacteria and metabolites is greater than in solid, therefore, the probability of encounter with the challenged bacteria will

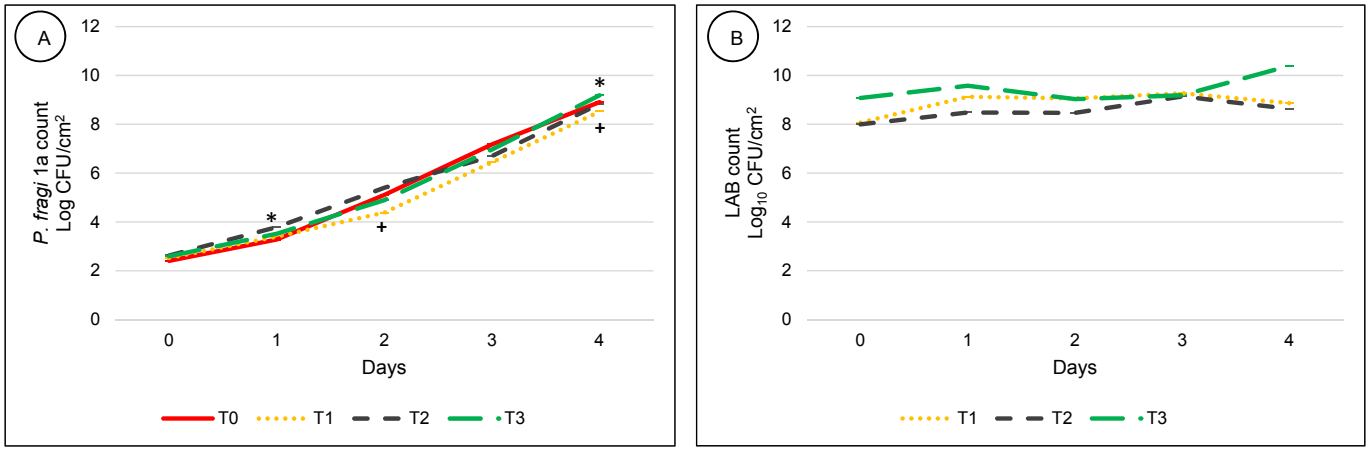

Fig. 3. *P. fragi* (A) and LAB (B) counts (mean ± SD Log_{10} CFU/cm^2) in *In situ* competitive inhibition in chicken meat at 8 ± 2 °C during 4-day storage period. *T0 (Red) Control treatment, ≈3 log_{10} CFU/cm^2 *P. fragi* without LAB (inoculated with NaCl 0,85%); T1 (Yellow), ≈3 log_{10} CFU/cm^2 *P. fragi* inoculated with ≈8 log_{10} CFU/cm^2 *L. sakei* L.A; T2 (Gray), ≈3 log_{10} CFU/cm^2 *P. fragi* inoculated with ≈8 log_{10} CFU/cm^2 *L. sakei* CECT 4808 L.B; T3 (Green), ≈3 log_{10} CFU/cm^2 *P. fragi* inoculated with ≈8 log_{10} CFU/cm^2 *L. rhamnosus* (L.D).*/+ indicate differences with statistical significance compared with control group (T0) ($p < 0.05$); *: Difference < 0.5 log_{10} UFC/cm^2; +: Difference > 0.5 log_{10} UFC/cm^2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

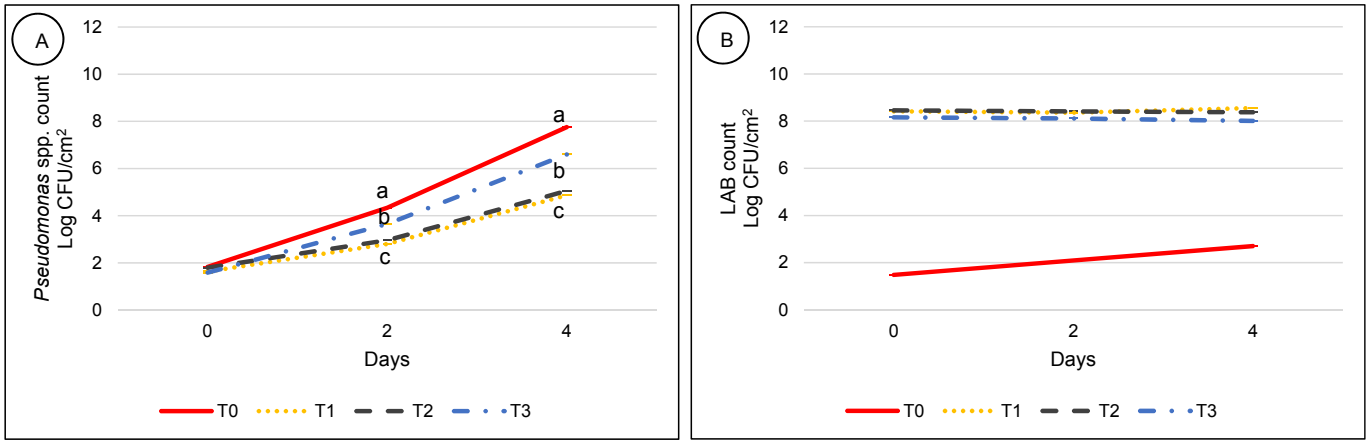

Fig. 4. *Pseudomonas* spp. (A) And LAB (B) counts (mean ± SD Log_{10} CFU/cm^2) in *In situ* competitive inhibition assay in fresh chicken meat at 8 °C during 4-day storage period.
*T0 (Red) Control treatment, chicken meat without LAB (inoculated with NaCl 0,85%); T1 (Yellow), chicken meat inoculated with ≈8 log_{10} CFU/cm^2 *L. sakei* L.A; T2 (Gray), ≈3 log_{10} CFU/cm^2 *P. fragi* inoculated with ≈8 log_{10} CFU/cm^2 *L. sakei* CECT 4808 L.B; T3 (Blue), chicken meat inoculated with ≈8 log_{10} CFU/cm^2 *Lactobacillus* sp. (L.C). Different letters indicate significant statistical differences between treatments by day ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

be greater. Blom et al. (1997) proved that the diffusion of some bacteriocins, like Nisin and Pediocin, on semi-solid agar is affected by the amount of fat, pH and NaCl of the medium. In contrast, in solid matrix, bacteria are more likely to live in physically structured habitats as colonies, and eventually, they could even form biofilms, such showed *P. fragi* is an initiator of biofilms (Sasahara & Zottola, 1993), protecting themselves from the action of any antibacterial substance (Galié, García-Gutiérrez, Miguélez, Villar, & Lombó, 2018).

In addition, bacteriocins stability is affected on meat. Fat can adsorb peptides, not leaving them available to exert their antagonistic effect. Also during cutting or grinding of raw meat, intracellular proteases are released to the meat surface, and therefore, the greater processing of meat (minced or ground) the lower is the stability or shelf life of bacteriocins (Favaro & Todorov, 2017).

In situ competitive inhibition assay, carried out in irradiated chicken meat at 8 °C is a simple test when sterile meat is available and may be closer to reality than *in vitro* assays with culture medium. Here, LAB antagonized targeted spoilage bacteria, both at defined concentrations on the proper matrix where the potential bio-preservative is to be applied. This reduce the uncertainty of an *in situ* assay in fresh meat, where species and, even strains, of poultry microbiota differ between produced batches (Morales et al., 2016a), therefore, the variability between experimental replicas could be higher. This work highlights the positive predictive value of this *in situ* test in irradiated meat, but not the negative one, since it could eventually rule out strains that have bio-preserving activity in fresh meat.

3.3. *In situ* protective cultures application in fresh chicken meat at 8 °C

In situ major bio-preservative potential in fresh chicken meat at 8 °C was evidenced with both *L. sakei* (T1 & T2), although neither grew during 4-day storage period (Fig. 4). Some minor antagonistic effect exert *Lactobacillus* spp. (T3) with *Pseudomonas* spp., in contrast with *in vitro* antagonistic assay in TSBYE, where no activity was evidenced against *P. fragi* (Fig. 2). Effect of all protective cultures tested on *Pseudomonas* spp. was observed from day 2 and remained on day 4, when the spoilage of the control meat (T0) was evident, both by microbial shelf life (*Pseudomonas* spp. > 7.5 Log CFU/cm^2) and for organoleptic characteristics (mainly odor) (Bruckner et al., 2012).

In this study, *in situ* raw poultry assays evidenced meat organoleptic changes that could be replicated in poultry sold in markets. Both L. *sakei* (T1 & T2) produced an acid smell, similar to butter, and superficial slime, which were not rejected by untrained personnel (5 laboratory technicians present at the time of analysis). Thus it would not be a problem for regular consumers. Samples inoculated with *Lactobacillus* spp. (T3) did not presented this aroma, but it was not evidently spoilage by odor by day 4. Castellano, González, Carduza, and Vignolo (2010) reported a similar odor in beef bio-preserved with *Lactobacillus curvatus*, which was not rejected by an expert panel. It is described that LABs in fresh meat produce a mild fermentation process, due to low carbohydrates content and strong buffering capacity of the meat, which does not produce obvious organoleptic variations (Favaro & Todorov, 2017).

Samples trans-illumination with UV light was useful to illustrate the bio-preservative effect of the LABs in fresh meat. Poultry treated with both *L. sakei* (T1 & T2), decrease the fluorescence attributable to some *Pseudomonas* species, probably *P. fluorescens* and *P. putida* (Palleroni, 2007), compared with those inoculated with *Lactobacillus* spp (T3) or uninoculated (T0) (Fig. 5).

To add to this study, greater bio-preservative potential observed in raw poultry assays, compared with irradiated poultry meat assays, could be explained by microbiota of raw meat, that is composed by microorganisms that interact with each other. Competition for essential nutrients, production of organic acids or other antimicrobial metabolites by some strains, negatively affect the survival or growth of other microorganisms (Huis in't Veld, 1996; Nychas et al., 2008). Møller et al. (2013) highlighted that high concentrations of natural microbiota in raw pork reduced growth of *Salmonella*. In contrast, in the sterile meat there is no microbial basal stressor; *P. fragi* did not have other competence to limit its growth than bio-preservant applied. In fresh chicken, effect of concomitant microbiota and inoculated lactic bacteria could act synergistically to delay growth of *Pseudomonas* spp.

In situ protective cultures application in fresh chicken meat had the advantage of emulate reality in small-scale, predicting the real bio-preservative potential that tested strains would have. However, a greater variability in the results could be expected due to the initial inoculum of the spoilage challenged bacteria, neither *Pseudomonas* species nor concomitant microbiota were handled.

4. Conclusion

All assays compared in this work (*In vitro* competitive inhibition in TSBYE and *In situ* in irradiated and fresh chicken meat) have some utility, advantages and disadvantages for the selection of lactic poultry bio-preservatives against spoilage microbiota.

In *in vitro* assay it is necessary to challenge a greater diversity of

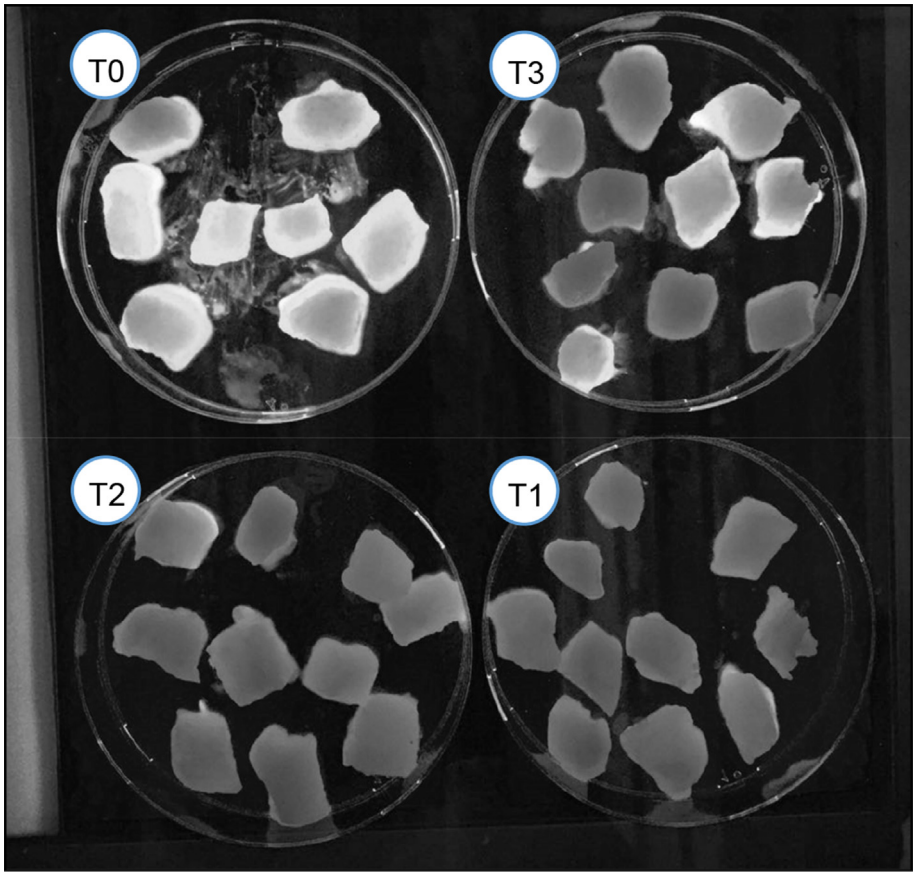

Fig. 5. Effect of bio-preservative cultures on poultry meat cubes (≈ 1 cm^3) observed under UV light on day 4 of storage in *in situ* competitive inhibition assay in fresh chicken meat at 8 °C. *T0 = uninoculated with BAL (+++ fluorescence), (−) control; T3 = *Lactobacillus* spp. L.C (+/- fluorescence); T2: *L. sakei* L.B (- fluorescence); T1: *L. sakei* L.A (- fluorescence).

bacterial strains to mimic species variability and reduce random uncertainty. Pre-selection of bio-preservatives using only one strain representing the great diversity of spoilage *Pseudomonas* spp., increase experimental error assay.

In *in situ* test on irradiated chicken meat, individual sensitivity of *P. fragi* does not necessarily represent the collective sensitivity shown by the *Pseudomonas* spp., immersed within a microbiota. *In situ* tests on irradiated meat should also incorporate the biological variability of challenged strains, and *In situ* assay on fresh poultry meat should never be dispensed with.

Definitive selection of bio-preservative must consider the real conditions in which the additive is to be used, since challenged bacteria diversity, concomitant microbiota and food matrix influence the antagonistic effectiveness of the lactic bacteria.

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