Original article
Bdellovibrio bacteriovorus to control *Escherichia coli* on meat matrices

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Summary   *Bdellovibrio bacteriovorus* is a predator micro-organism towards other Gram-negative bacteria. We tested *B. bacteriovorus* to control *Escherichia coli* growth on chicken slices and canned beef. Moreover, we analysed *B. bacteriovorus's* lytic ability on eight toxigenic or multidrug-resistant *E. coli* strains. In chicken slices, the predator induced the highest prey reduction (4.3 log) respect to control at 6 h. In canned beef, the predator induced the highest prey reduction (2.1 log) respect to control at 6 h. Moreover, *B. bacteriovorus* showed lytic ability towards all tested *E. coli* strains. *B. bacteriovorus* could control *E. coli* and other pathogenic and spoilage bacteria in those meat-based foods that have a shelf life <10 days. It could integrate modified atmosphere packaging (MAP) to prolong the shelf life and improve the safety of pre-packed fresh meat, meat preparations and meat products. In future applications on meat-based foods, *B. bacteriovorus* could also minimise the use of additives.

Keywords   *Bdellovibrio* and like organisms, *Bdellovibrio bacteriovorus*, *E. coli*, STEC.

Introduction

Bdellovibrio bacteriovorus belongs to the group of *Bdellovibrio* and like organisms (BALOs) that are Gram-negative, aerobic bacteria predatory towards other Gram-negative bacteria (Stolp & Starr, 1963; Williams *et al.*, 2005). BALOs have been the focus of research for nearly five decades, with exciting practical applications to medical, agriculture, veterinary and for treatment of human pathogenic and drug-resistant bacteria (Dwidar *et al.*, 2012; Kadouri *et al.*, 2013). In particular, *B. bacteriovorus* is a predator which attacks many microorganisms and its favourite prey is *Escherichia coli,* including commensal and pathogenic as Shiga toxin-producing strains (STEC) (Fratamico & Whiting, 1995). As it has been widely demonstrated that *B. bacteriovorus* cannot grow in eukaryotic cells (Dwidar *et al.*, 2012), it does not represent a specific risk for the safety of humans. *B. bacteriovorus* has been isolated from intestine of vertebrates, including humans (Dwidar *et al.*, 2012; Iebba *et al.*, 2013). In humans, it is abundant only in healthy subjects and it is therefore considered a member of the common intestinal microbiota with a key role in maintaining health (Iebba *et al.*, 2013). *B. bacteriovorus* does not carry genes of antibiotic resistance (Shemesh & Jurkevitch, 2004; Dwidar *et al.*, 2012). *B. bacteriovorus* do not prey on those useful Gram-positive bacteria or fungi, such as lactic acid bacteria, staphylococci, yeasts, which play the role of starter cultures for some food products (Dwidar *et al.*, 2012). *B. bacteriovorus* could reduce both spoilage and pathogens on the food. Finally, its capability to parasitise bacteria organised in biofilms or in viable but non-culturable (VBNC) forms (Kadouri & O'Toole, 2005; Markelova, 2010; Dashiff *et al.*, 2011; Dwidar *et al.*, 2012), make *B. bacteriovorus* not susceptible to those common mechanisms of competition or defence that the pathogens and spoilage bacteria could activate on foods. Biofilms of spoilage and pathogenic microflora that form on contact surfaces are often responsible for the contamination of food during post-processing production. It has been shown that, even with diligent cleaning and sanitation, microorganisms within biofilms can remain viable on equipment surfaces (Wang *et al.*, 2015; Al-Adawi *et al.*, 2016). Biological treatments involving

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bacteriocins and bacteriophages have been widely investigated for pathogen inactivation in fresh meat (Zhou et al., 2010; Castellano et al., 2017). When compared to other biological tools, such as bacteriophages, *B. bacteriovorus* and other BALOs show a very non-specific predation of their host cells. Consequently, *B. bacteriovorus* can attack Gram-negative bacteria from very distinct and different genera (Fratamico & Whiting, 1995; Dwidar et al., 2012). Preservation technologies for foods are mainly based on refrigeration, modified atmosphere packaging (MAP) and addition of chemical additives (Zhou et al.,2010; Castellano et al., 2017). New technologies such as high hydrostatic pressure, superchilling, natural bio-preservatives and active packaging have been proposed (Zhou et al., 2010; Castellano et al., 2017). To date, as live protective cultures to be applied in foods and food processing surfaces, BALOs have only been explored to a limited degree (Fratamico & Cooke, 1996; Lu & Cai, 2010). *B. bacteriovorus* was never applied on meat matrices. It could find application in containing spoilage and pathogenic bacteria in meat-based foods substituting or integrating conventional approaches. Challenge experiments, in which *B. bacteriovorus* and prey were made to interact in a liquid medium and their growth monitored over time, have been performed in the past decades (Fratamico & Whiting, 1995). The predatory efficacy was influenced by pH, incubation temperature and predator/prey ratio (Fratamico & Whiting, 1995).Various other parameters with respect to the liquid medium can influence the predator's performances on a meat matrix, such as the residential microbial community, the physical–chemical characteristics and the storage temperature. The availability of oxygen and water activity (Aw) could also influence the predator's ability on a meat matrix. These last two factors depend very much on the way in which the meat is contaminated with the predator, that is on the surface or in the mass. This is a preliminary study to evaluate the efficacy of *B. bacteriovorus* to prey *E. coli* on two meat matrices which have been contaminated with the two different modalities. In this phase, we did not consider the possible role of the residential microbial community, the physical–chemical properties and the real storage conditions of these meat-based foods. Then, *B. bacteriovorus* and *E. coli* were spread on surface of sterilised chicken slices and inoculated in the mass of canned beef and their growth monitored at 30 °C ± 1 °C at 0, 1, 3, 6, 24 h . To understand the factors that could influence the predator/prey relationship in the tested meat matrices, we performed in parallel challenge experiments in liquid medium under the same conditions of pH, temperature and predator/prey ratio. Moreover, we tested *B. bacteriovorus* lytic ability on eight STEC or multidrug-resistant *E. coli* strains.

Materials and methods

Collection and field strains used

For challenge experiments on meat matrices *E. coli* ATCC 15144 was used as prey. *E. coli* enrichments were prepared from a stock culture grown on brain heart infusion broth (BHI Difco; Becton, Dickinson and Company, Milano, Italy) until prey reached an OD_{600} of 0.80 (~1.8×10^7 CFU per mL) (Richards et al., 2016). *B. bacteriovorus* 109 ATCC 15143 was used as predator. To prepare the attack-phase of the predator, 20 µL of the predator stock culture and 100 µL of the prey enrichment were added to 10 mL of dilute nutrient broth (DNB; Difco; Becton, Dickinson and Company, Milano, Italy) and incubated at 30 °C ± 1 °C (Fratamico & Whiting, 1995). One-day enrichments of predator (approximately 1×10^9 PFU per mL) were filtered through a 0.45- m-pore-size Millex HV syringe filter (Millipore Corp., Billerica, MA) to remove primary prey.

Preliminary study to evaluate the optimal prey–predator ratio to be used in challenge experiments

Prior to each experiment, predator and prey enrichments were quantified. For predator, a double layer agar plating assay, at 30 °C ± 1 °C, was performed. For each assay, 25 mL of modified polypeptone peptone (Pp) plus bacto agar (Pp20; Biolife, Milano, Italy) was dispensed into a 100-mm Petri dish and allowed to harden so as to make a bottom layer. Then, 1 mL of filtered enrichment and its 10-fold serial dilutions in DNB and 0.1 mL of OD_{600} of 0.80 prey culture were inoculated in 17 mL of molten (48 °C) Pp20 (Ottaviani et al., 2018). The tubes were inverted three times to mix and poured on top of the bottom layer and the plates were incubated at 30 °C ± 1 °C for 24 h until 5 days (Ottaviani et al., 2018). For prey, 1 mL of undiluted enrichment and its 10-fold serial dilutions in peptone salt solution were inoculated onto tryptone-bile-glucuronic medium agar (TBX; Biolife, Milano, Italy) and the plates were incubated at 44 °C ± 1 °C for 24 h. The counts of predator and prey ranged from 1.8 to 3.5×10^9 PFU per mL and from 1.5 to 3.0×10^7 CFU per mL. Previously, it has been reported that *B. bacteriovorus* was most effective in lysing *E. coli* at levels of 10^9 CFU per mL, in liquid medium (Fratamico & Whiting, 1995). However, we decided to test prey levels lower than those tested by Fratamico but that could reflect the real *E. coli* contamination of environment and meat-based foods (Cassin et al., 1998; Jang et al., 2017). Moreover, since *bdellovibrios* encounter susceptible prey bacteria by random collision (Fratamico & Whiting, 1995), we increased by 1–2 log the level of the parasite,

compared to that of prey, to optimise the attack rate. In the light of this, the test flask containing 10 mL of DNB were inoculated with the following predator/prey concentrations: 10^5 PFU/ 10^3 CFU per mL, 10^6 PFU/ 10^4 CFU per mL, 10^7 PFU/ 10^5 CFU per mL, 10^7 PFU/ 10^6 CFU per mL, 10^8 PFU/ 10^6 CFU per mL, 10^9 PFU/ 10^7 CFU per mL. The same prey concentrations were inoculated into DNB, without predator, as controls. Cultures were incubated at 30 °C ± 1 °C on a shaker. To count the prey in the test and control, 1 mL of DNB and its 10-fold serial dilutions were inoculated in duplicate in TBX at 0 and 6 h and the plates incubated at 44 °C ± 1 °C for 24 h.

Challenge experiments in meat matrices

Chicken breast slices packaged in modified atmosphere (MAP) were purchased from the market and then were further cut into 20 pieces, each weighing 10 g. The pieces were placed in glass jars and autoclaved for 15 min at 121 °C ± 1 °C. They were randomly assigned to control and test, with 10 fillets each. Prior the experiment, pH (pH meter 300 Hanna Instruments, Milan, Italy) and Aw (Dew Point Water Activity meter, Aqua Lab Decagon Devices, Steroglass Decagon Distributer, Perugia, Italy) were measured on an another 100 g piece and they were 6.10 and 0.99. The 10 g pieces in control and test were contaminated by spreading 0.5 mL of 10^5 per mL prey suspension onto the entire surface on one side of each 10 g piece. They were left to aerate in the laminar flow cabinet for 15 min. Then, test portions were contaminated by spreading 0.5 mL of 10^7 PFU per mL predator. For control portions, the same amount of peptone salt solution was used. All portions were incubated at 30 °C ± 1 °C and counts at 0, 1, 3, 6 and 24 h after treatments were performed. For prey, 10 g were 1:10 diluted in buffered peptone water, homogenised and serially diluted in peptone salt solution (ISO 6887-1, 2017). Then, 1 mL of each dilution was inoculated onto TBX in duplicate and the plates incubated at 44 °C ± 1 °C for 24 h (ISO 16649-2, 2001). BALOs counts by plaque assay were performed combining 0.1 mL of prey culture (at an OD_{600} of 0.80) and 1 mL of undiluted or diluted meat homogenate and the plates incubated at 30 °C ± 1 °C for 24 h until 5 days (Ottaviani et al., 2018). The data were plotted as plaque-forming units per gram piece (PFU per g). Canned beef in packets of 140 g, purchased from the market had the following composition: beef, broth, salt, gelling agent, agar agar, thickener, carob seed flour, flavour enhancer and sodium nitrite. The meat was extracted from packets and divided into 20 blocks each weighing 10 g and placed in as many sterile glass jars. They were randomly assigned to control and test with 10 blocks each. Prior to the experiment, pH and Aw were measured on another 100 g block and they were 6.09 and 0.98. The 10 g blocks in control and test were homogeneously contaminated inoculating by a syringe 0.5 mL of 10^5 per mL *E. coli* suspension and 0.5 mL of 10^7 PFU per mL of predator in each test block. For the control portions, the same amount of peptone salt solution was used. Predator and prey counts were carried out as for the chicken slices. For both experiments, in parallel, analogous DNB test and control were prepared and analysed at the same meat pH, temperature of incubation, predator/prey ratio.

Lytic ability of *B. bacteriovorus*

Lytic ability of one-day filtered *B. bacteriovorus* enrichments was determined by monitoring its ability to form clear lytic halos on a lawn of STEC or multidrug-resistant *E. coli* preys (Table 1). Briefly, 1 mL of filtered predator undiluted enrichment and 0.1 mL of 0.80 OD_{600} prey were assayed using a double layer agar plating technique. Plates were incubated for 1 to 5 days at 30 °C ± 1 °C. Prey bacteria were regarded as being susceptible if any clear plaques appeared on the double layer agar plates; and unsusceptible if otherwise (Ottaviani et al., 2018). For each prey specificity assay, a positive control, represented by primary prey and predator and a negative control, which consisted of primary prey alone, were performed.

Table 1 Preys assayed in this study with *Bdellovibrio bacteriovorus* 109

Prey strains	Origin	Toxin genes *Drug resistances [†]	Susceptibility to *B. bacteriovorus*
E. coli	ATCC 25922	− [‡]	+ [§]
E. coli	Food origin, Italy	AMP, C, AMC, SH	+
E. coli O111	EURL[¶]	*eae*	+
E. coli O103	EURL	*eae, stx2*	+
E. coli O157	EURL	*eae, vtx1, vtx2*	+
E. coli O26	EURL	*eae, stx1, stx2*	+
E. coli O145	EURL	*eae, stx1*	+
E. coli O104 H:4	EURL	*aggR, aaiC*	+
E. coli O157 H7	ATCC 35150	*stx1, stx2*	+

*stx1: Shiga toxin 1 gene; *stx2*: Shiga toxin 2 gene; *eae*: intimin gene; *vtx1*: verocytotoxins 1 gene; *vtx2*: verocytotoxins 2 gene; *aggR*: transcriptional activator gene; *aaiC*; secreted protein gene.
[†]Ampicillin (AMP 10 μg); chloramphenicol (C 30 μg); amoxicillin + clavulanic acid (AMC 20/10 μg spectinomycin (SH).
[‡]Negative.
[+]Positive.
[¶]EU Reference Laboratory for *E. coli* (EURL).

Statistical analysis

Challenge experiments in meat matrices and liquid medium were repeated by three separate trials and each trial was carried out in duplicate ($n = 6$). Results of microbiological analyses were reported as mean values (log transformed) ± standard deviation. Means of plate counts were analysed for differences in response to predator treatments using Student's t-test. Statistical calculations were based on confidence level equal to or higher than 95%. Preliminary study to evaluate the optimal prey–predator ratio to be used in challenge experiments was performed in duplicate and means (log-transformed) of prey counts were calculated.

Results and discussion

Preliminary study to evaluate the optimal prey–predator ratio to be used in challenge experiments

The difference in the prey counts of duplicate experiments was always within 0.5 log units. The results are shown in Table 2. Although the levels of prey we tested were lower than those considered optimal to induce the maximum predatory activity of *B. bacteriovorus*, all concentrations, except 10^3 CFU per mL, induced a detectable predatory activity with significant lower prey level in the test than in the control. Under the tested conditions, the highest prey reduction equal to eight log was obtained for predator/prey ratio of 10^7 PFU/10^5 CFU per mL.
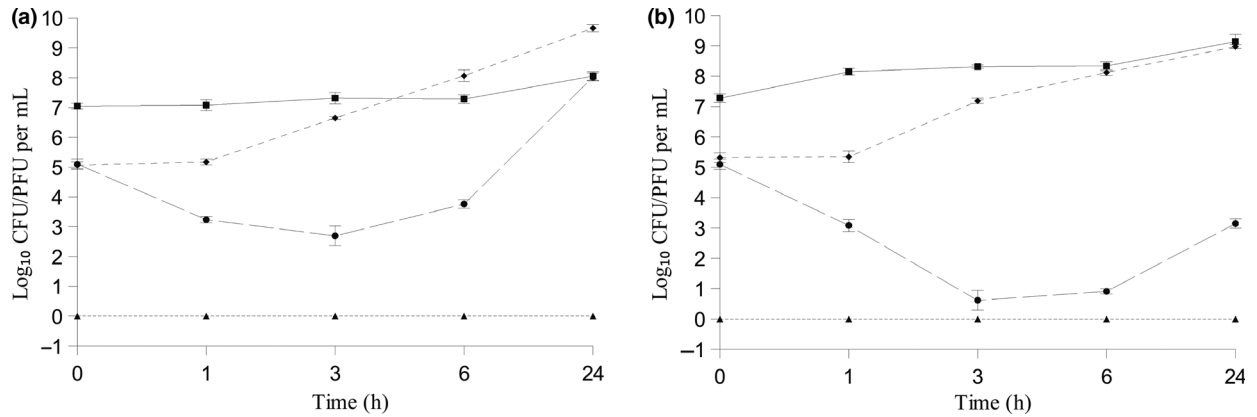
Challenge experiments in meat matrices

Results of the experiment in chicken slices are shown in Fig. 1. In the meat test, predator concentration remained at the same level from 0 to 6 h, then it increased by about 1 log, from 10^7 to 10^8 PFU per g, between the 6 and 24 h. In the meat control, predator counts were always not detectable (<10 PFU per g) from 0 to 24 h. By comparing predator concentrations

Table 2 Effectiveness of *Bdellovibrio bacteriovorus* 109 at reducing the level of *Escherichia coli* after 6 h at 30 °C

PFU predator/ CFU prey per mL	Log prey increase between 0 and 6 h		Log prey reduction in test respect to control at 6 h
	Test	Control	
10^9/10^7	−4.00	0.53	4.53
10^8/10^6	−1.7	2.50	4.20
10^7/10^5	−6.00	2.00	8.00
10^7/10^6	−1.48	2.42	3.90
10^6/10^4	−2.48	4.42	6.90
10^5/10^3	1.7	4.00	2.30

at different time points in meat and DNB tests, higher *B. bacteriovorus* levels for all time points in DNB than in chicken slices were observed, with the maximum significant difference (1.1 log) at 24 h. By comparing prey concentrations at different time points in the meat test and control, significantly lower *E. coli* levels for all time points were observed in the test than in the control, with the maximum difference (4.3 log) at 6 h. Prey counts obtained at different time points were significantly lower in the DNB test than in the meat test from 3 to 24 h. The maximum difference (4.9 log) was observed at 24 h. The results of the challenge experiment in canned beef are shown in Fig. 2. In the meat test, the predator concentration increased by about 0.5 log from 0 to 1 h, then it decreased by about 1 log from 1 to 3 h. It remained on the same level from 3 to 6 h, and finally it increased by about 0.5 log, from 6 to 24 h. In the meat control, the predator counts were always not detectable (<10 PFU per g) from 0 to 24 h. Predator concentrations at different time points in meat and DNB tests were significantly higher at all time points in the DNB than in the meat, with the maximum difference (1.9 log) at 3 h. By comparing prey concentrations at different time points in the meat test and control, significantly lower *E. coli* levels were observed for all time points in the test than in the control, with the maximum difference (2.1 log) at 6 h. Prey counts at different time points were significantly lower in the DNB than in the meat test from 3 to 24 h. The maximum difference (5.1 log) was at 6 h. Using a predator/prey ratio of 10^7 PFU/10^5 CFU per g *B. bacteriovorus* was able to contain the prey level growth in both meat tests, with a significant reduction in respect to the controls after 1 h. *B. bacteriovorus* preys *E. coli* more effectively in the liquid medium than in the meat matrices, under the same experimental conditions A possible explanation to this could be that the loss of water content and/or lack of water film on meat foods made these less suitable than broth for *B. bacteriovorus* predatory activity (Sockett, 2009). This hypothesis is strengthened by the predator trend in meat and DNB tests, with predator counts always lower in first than in the second, for the entire analysis period. The greater predator efficiency of killing prey obtained in the chicken slices than in canned beef may depend, at least partially, on the experimental contamination mode. In fact, predator and prey were spread on surface of chicken slices and inoculated in the mass of canned beef. As a result, the predator had a greater possibility of swimming and availability of oxygen in the first matrix than in the second one. A previous report examined potential of another member of BALOs that is *Perdibacter* in controlling growth of *Salmonella* on tilapia in fillets (Lu & Cai, 2010). The authors observed that the efficiency of *Peridibacter* towards *Salmonella* was conditioned by the viscosity
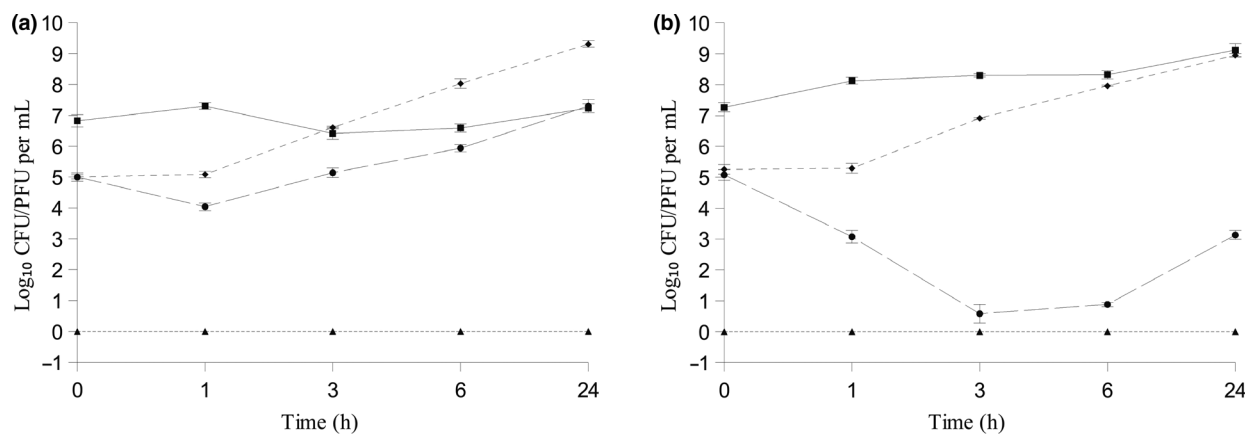
Figure 1 Challenge experiment in chicken slices. The population dynamics of *Bdellovibrio bacteriovorus* (Bb) and *Escherichia coli* in test (with Bb) and control (without Bb) meat (a) and dnb (b). ♦ *E. coli* control; ● *E. coli* test; ▲ Bb control; ■ Bb test.

of the prey / predator suspension used to contaminate the food. They hypothesised that the excessive viscosity of the medium hindered *Peridibacter* in swimming and attacking the prey. Previously, it has been reported that *B. bacteriovorus* preyed *E. coli* in liquid medium between 12 ° C and 37 °C, with the maximum activity in the first 7 h (Fratamico & Whiting, 1995; Fratamico & Cooke, 1996). In the light of these evidences and our preliminary results, we believe that *B. bacteriovorus* could be a potential candidate to be used in meat foods with a short shelf life (<10 days), such as fresh meat, meat preparations and meat products, including some types of RTE. In these products, *B. bacteriovorus* could be spread on the surface (for example on fresh meat in slices or blocks) or inoculated in the mass (for example in minced meat and fresh sausages) during the preparation. It could exert a

prey reduction in the time interval comprising the preparation and the MAP, before storage at 4 °C. During storage, prey levels could be further contained by low temperature and the MAP.

Lytic ability of *B. bacteriovorus*

All six repeated experiments on each prey strain gave consistent results. *B. bacteriovorus* had the ability to attack all tested preys. For all preys, plaques of lysis became visible after 24 h and then, their sizes expanded over time, reaching the maximum after 3 days of incubation at 30 °C ± 1 °C. At the end of the incubation, diameters of lysis were similar to those on primary prey, ranging between 7 and 9 mm. In our challenge experiments, we tested *B. bacteriovorus* with a non-pathogenic type strain of *E. coli;* however,

Figure 2 Challenge experiment in canned beef. The population dynamics of *Bdellovibrio bacteriovorus* (Bb) and *Escherichia coli* in test (with Bb) and control (without Bb) meat (a) and DNB (b). ♦ *E. coli* control; ● *E. coli* test; ▲ Bb control; ■ Bb test.

previous work had shown that growth rates of pathogenic and non-pathogenic *E. coli* are similar (Cassin *et al.*, 1998). In the light of this, and since *B. bacteriovorus* showed lytic ability towards all tested preys, it is conceivable that similar predation on meat matrices could also be exercised with respect to STEC for which the meat represents one important source of infection (Caprioli *et al.*, 2015; Jang *et al.*, 2017). Our laboratory-scale objectives over the next few years will be to (a) test *B. bacteriovorus* towards different food-borne pathogenic *E. coli* strains, natural contaminating spoilage bacteria and bacteria included in process hygiene and safety criteria of EC Regulation No 2073 (2005) (b) test *B. bacteriovorus* towards prey clones to be easily distinguished by selective agar media on meat-based foods stored at 4 °C.

Conclusion

This is the first application of *B. bacteriovorus* as live protective cultures towards *E. coli* on meat matrices. In the light of our results, *B. bacteriovorus* for its biological properties and predation mode, could represent a suitable candidate for the development of new biological strategies to control *E. coli* and other pathogenic and spoilage bacteria in those meat-based foods that have a shelf life <10 days. *B. bacteriovorus,* as competitive micro-organism, could integrate MAP to prolong the shelf life and improve the safety of pre-packed fresh meat, meat preparations, meat products, including some types of RTE. In future applications on meat-based foods, *B. bacteriovorus* could also minimise or avoid the use of additives, particularly preservatives and antioxidants.

Acknowledgments

Ethics approval

Ethics approval was not required for this research.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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