Effectiveness of *Bdellovibrio bacteriovorus* to contain *Escherichia coli* on milk and temperature impact on predation dynamics

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PAPER

Abstract

We tested the predation of *B. bacteriovorus* against *Escherichia coli* in milk samples in three different experiments. In Experiment 1, the growth and predatory activity of *B. bacteriovorus* against *E. coli* in milk stored at 4°C were evaluated. In Experiment 2, the predatory activity of *B. bacteriovorus* against *E. coli* in the milk matrix was compared to the optimal one in the medium of choice. In Experiment 3, the influence of the native milk microbial community on the predation of *B. bacteriovorus* against *E. coli* experimentally added or indigenous grown at 4°C was tested. The predator increased at 4°C by about 1 Log in the first 48 hours and caused *E. coli* decrease by about 2 Log after 24 hours. The predator at 30°C reduced *E. coli* faster (3 Log after 6 hours) than at 4°C (2 Log after 24 hours). *B. bacteriovorus* at 30°C preyed on *E. coli* more in the nutrient broth than in the milk, with the most significant difference by about 4 Log after 48 hours. In raw milk contaminated only by the predator, it increased by about 1 Log after 48 hours at 4°C, suggesting that it preyed on indigenous microorganisms. *B. bacteriovorus* could find application in raw milk used as food or raw material during storage at 4°C to reduce the microbial load of spoilage and Shigatoxin-producing *E. coli* (STEC) strains of *E. coli* and therefore increase its shelf-life and healthiness.

Keywords: *Bdellovibrio* and like organisms; *Bdellovibrio bacteriovorus*; *E. coli*; milk

Introduction

In Italy and other industrialized countries, the consumption of raw milk has been increasing in recent years due to the growing interest of consumers in untreated and locally produced foods (Tremonte *et al*., 2014). Indeed, the consumption of raw milk may have a protective association with the development of allergies and it is an important source of vitamin B2 (Macdonald *et al*., 2011). However, the presence of pathogenic microorganisms in raw milk is reported everywhere (Boor *et al*., 2017; Macdonald *et al*., 2011). The legislation dictates to store raw milk at 4°C for 72 hours and to consume it only after boiling to ensure its safety (EU, 2004; Italian Ministry of Health, 2009, 2013). However, the responsibility to sanitize raw milk lies with the consumer, who may underestimate or ignore the microbiological risks associated with it (Tremonte *et al*., 2014). Moreover, when milk is used as a raw material to produce pasteurized, UHT (Ultra High Temperature) milk, and other milk-based foods,

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its refrigeration for up to 72 hours is the most efficient method to increase its shelf life and eliminate spoilage by mesophilic bacteria (Boor *et al*., 2017). However, refrigeration of raw milk does not limit the development of psychrotrophic bacteria like *Pseudomonas* (Boor *et al*., 2017). These microorganisms produce thermostable extracellular enzymes that can affect the quality of milk and dairy products after heat treatments when exceeding 10^6 CFU (Colony-Forming Unit) per mL (Boor *et al*., 2017). To increase the safety and microbiological quality of raw milk, biological control methods based on bacterium-bacterium predation could be tested. *Bdellovibrio* and like organisms (BALOs) are gram-negative, aerobic bacteria that are predatory toward other gram-negative bacteria (Stolp and Starr, 1963; Williams *et al*., 2005). BALOs have been investigated over the past 50 years with promising results in medicine, agriculture, veterinary, and for the treatment of pathogenic and drug-resistant bacteria (Dwidar *et al*., 2012; Kadouri *et al*., 2013). Compared to bacteriophages, BALOs show more non-specific predation as they can attack gram-negative bacteria of distinct genera (Dwidar *et al*., 2012; Fratamico and Whiting, 1995). In particular, the favorite prey of *Bdellovibrio bacteriovorus* is *Escherichia coli,* including commensal and pathogenic as Shigatoxin-producing *E. coli* strains (STEC) (Fratamico and Whiting, 1995; Ottaviani *et al*., 2019). However, it is also able to attack other pathogenic and spoilage bacteria such as *Salmonella*, *Shigella, Yersinia, Serratia*, *Proteus*, *Pseudomonas* (Dashiff *et al*., 2011). *B. bacteriovorus* does not pose a risk to humans as it does not grow in eukaryotic cells (Bratanis *et al*., 2020; Dwidar *et al*., 2012). *B. bacteriovorus* does not carry harmful or antibiotic resistance genes and does not prey on gram-positive bacteria or fungi playing the role of starter cultures for some food products (Dwidar *et al*., 2012; Shemesh and Jurkevitch, 2004). Finally, *B. bacteriovorus* can prey on bacteria even if organized in biofilm or viable but non-cultivable (VBNC) (Dashiff *et al*., 2011; Dwidar *et al*., 2012; Kadouri and O'Toole, 2005; Markelova, 2010). Previous research has shown that pH, incubation temperature, predator/prey ratio, and the physical and chemical characteristics of the medium can influence predation (Fratamico and Whiting, 1995; Sockett, 2009). Most of the published studies have used pure cultures in liquid medium to elucidate the prey-predator interaction. In those experimental contexts, *B. bacteriovorus* showed preferential predation on *E. coli*, but the basis for this selection is not known. The interest of the scientific community and legislative bodies in these microorganisms has been increasing in recent years. Recently, for the first time, experts from the European Food Safety Agency (EFSA) have proposed the use of predatory bacteria such as *B. bacteriovorus* as one of the measures to be taken in food production environments to contain antimicrobial resistance (EFSA, 2021). Moreover, the United States Department of Agriculture (USDA) is beginning to test *Bacteriovorax* and *Bdellovibrio* for bio-based food sector intervention strategies (USDA, 2018). However, these lines of study have not yet produced published data, to the best of our knowledge. To date, the only data available are those concerning the application of *B. bacteriovorus* on some sterilized foods and food processing surfaces on a laboratory scale (Fratamico and Cooke, 1995; Lu and Cai, 2010; Ottaviani *et al*., 2019; Youdkes *et al*., 2020). The predatory ability of *B. bacteriovorus* in complex natural habitats, with mixed microbial flora, such as raw milk, is yet to be discovered. This study is the first application of *B. bacteriovorus* as a predator on milk to obtain laboratory-scale information on its predation potential on a food matrix never tested before. In particular, it was investigated how the refrigeration, "milk matrix," and the indigenous microbial community could influence the predation of *B. bacteriovorus* against its preferred prey, that is, *E. coli.*

Materials and Methods

Collection and field strains used

E. coli ATCC 15144 was used as prey. *B. bacteriovorus* 109 J ATCC 15143 was used as a predator. *E. coli* enrichment and the attack phase of the predator were prepared according to previously standardized methods (Ottaviani *et al*., 2019). For the challenge experiments, we used the predator/prey ratio of 10^7 PFU/10^5 CFU per mL (PFU=Plaque-Forming Unit) to activate the best predation (Ottaviani *et al*., 2019).

Milk sampling

Each sample was 1460 mL of raw milk from Marche's breed cows from an automatic raw milk machine inside a dairy farm located in the Ancona province (The Marches Region, Central Italy). Samples were stored in a cool box at 4°C and tested within 2 hours from the sampling. Four batches were made from each sample, one of 540 mL and three of 300 mL. Before the experiment, on other 20 mL of each sample pH (pH meter 300 Hanna Instruments) was measured, and it ranged between 6.68 and 6.88. Raw milk from the same machine had been analyzed for indigenous *E. coli,* according to the standard method (ISO 16649-2:2001). In the previous 6 months, *E. coli* counts never exceeded 4 Log CFU per mL.

Experiment 1

A 540 mL volume of the first batch was poured into a sterile container and heated until foam appeared, then the heating was stopped. Immediately after boiling, the

milk was dispensed into 54 tubes, each with 10 mL of milk. Then, 27 tubes were assigned to the control and the same number to the test. To obtain in milk a predator/prey ratio of 10^7 PFU/10^5 CFU per mL, all tubes were homogeneously contaminated by inoculating 0.5 mL of prey suspension at a concentration of about 5×10^6 CFU per mL. The test tubes were then contaminated with 0.5 mL of about 5×10^8 PFU per mL predator. The same amount of peptone salt solution was added to the control. All tubes were stored at 4°C. Predator and prey counts were performed at 0, 6, 24, 48, 72, 96, 120, 144, 168 hours from the treatment (Table 1). The predator was also tested in the milk control to exclude a cross-contamination with the predator experimentally added to the milk test. For the prey enumeration, 9 mL were diluted 1:10 in buffered peptone water and from the stock suspension, decimal dilutions in peptone salt solution were made (ISO 6887-1:2017). Then 1 mL of each dilution was inoculated on Tryptone Bile X-GLUC Agar (TBX) (Biolife, Milan, Italy) in duplicate, and the plates were incubated at 44°C for 24 hours (ISO 16649-2:2001). The data were reported as CFU per mL. *B. bacteriovorus* enumeration was performed with the plaque assay combining 0.1 mL of prey enrichment and 1 mL of undiluted or diluted milk, and plates were incubated at 30°C for 24 hours up to 5 days (Ottaviani *et al.*, 2019). The data were reported as PFU per mL.

Experiment 2 (A, B)

The second batch was heated as in Experiment 1. Immediately after boiling, the milk was dispensed into 30 tubes, each with 10 mL. Fifteen tubes were assigned to the control and the same number to the test. All tubes were homogeneously contaminated as described for Experiment 1 and stored at 30°C. The predator and prey counts were performed at 0, 6, 24, 48, 72 hours from the treatment as in Experiment 1 (Experiment 2A). In parallel, an analogous test and control were prepared, represented by the medium of choice for predator (2B) that was diluted nutrient broth (DNB) consisting nutrient broth (Biolife, Milan, Italy) at a concentration of 0.08 g per liter of distilled water (Experiment 2B) (Table 1).

Experiment 3 (A, B)

An experiment analogous to Experiment 2A was performed on the third batch at 4°C (Experiment 3A).

Table 1. Predator/prey challenge experiments

Experiment number and aim		Experimental design		Storage T°C	Timescale predator/prey counts—hours
1	Evaluate growth and predatory activity of *B. bacteriovorus* against *E. coli* in milk stored over time up to 7 days.	**Milk test**: Boiled milk contaminated with the predator/prey ratio 10^7 PFU/10^5 CFU **Milk control**: Boiled milk contaminated with 10^5 CFU prey		4	0, 6, 24, 48, 72, 96, 120, 144, 168
2 (A, B)	Evaluate predatory activity of *B. bacteriovorus* against *E. coli* in milk matrix: (**A**) during the shelf-life of raw milk; (**B**) compared to DNB, the medium of choice.	**A** **Milk test**: Boiled milk contaminated with the predator/prey ratio 10^7 PFU/10^5 CFU **Milk control**: Boiled milk contaminated with 10^5 CFU prey	**B** **DNB test:** DNB contaminated with the predator/prey ratio 10^7 PFU/10^5 CFU **DNB control:** DNB contaminated with 10^5 CFU prey	30	0, 6, 24, 48, 72
3 (A, B)	Evaluate influence of the native milk microbial community on the predation of *B. bacteriovorus* against *E. coli* during the shelf-life of raw milk: (**A**) by adding *E. coli* experimentally under the best prey/predator ratio; (**B**) at natural concentrations of *E. coli*.	**A** **Milk test**: Raw milk contaminated with the predator/prey ratio 10^7 PFU/10^5 CFU **Milk control**: Raw milk contaminated with 10^5 CFU prey	**B** **Milk test**: Raw milk contaminated with 10^7 PFU predator **Milk control:** Raw milk	4	0, 6, 24, 48, 72

DNB, diluted nutrient broth. Graphical scheme of performed experiments: 1, 2 (A, B), 3 (A, B).

From the microbiological analyses of raw milk carried out in the previous 6 months indigenous *E. coli* had never reached 5 Log, which is the optimal level for predation at maximum efficiency (Ottaviani *et al*., 2019). For this reason, the milk sample was contaminated with 5 Log *E. coli* ATCC 15144. In Experiment 3B, the growth of *B. bacteriovorus* and its predation toward natural level of indigenous *E. coli* were evaluated in raw milk, under normal storage conditions, for a time corresponding to its shelf-life. Then on the fourth batch, an experiment on raw milk contaminated only with the predator at a concentration of 10^7 PFU per mL was performed at the same conditions as Experiment 3A (Table 1).

Statistical analysis

Each experiment was repeated in three separate trials, and each trial was carried out in triplicate (n = 9). Results of microbiological analyses were reported as mean values (Log-transformed) ± standard deviation. Mean of plate counts were analyzed for differences in response to predator treatments using the Student's t-test (t). Statistical calculations were based on confidence levels (P) equal to or higher than 95%.

Results and Discussion

Experiment 1

In this experiment, the influence of raw milk storage temperature on the growth of *B. bacteriovorus* and its predation ability toward *E. coli* was evaluated. Then, we tested boiled milk contaminated with the predator/prey ratio to get the best predation at 4°C. Predator and prey counts were performed at 0, 6, 24, 48, 72, 96, 120, 144, 168 hours from the treatment in test and control. The results are summarized in Figure 1. The optimal growth temperature of *B. bacteriovorus* is between 28 and 30°C, making it a mesophile (Stolp and Starr, 1963; Williams *et al*., 2005). However, in this experiment, the predator multiplied from 24 to 72 hours and then held the experimentally added charge until the end of the experiment at 4°C. Lower *E. coli* levels of the test with respect to the control were observed for all time points, with the greatest significant difference of about 2 Log after 24 hours (t = 12.3747; P < 0.0001). Previous studies have shown that *B. bacteriovorus* preyed on *E. coli* in the liquid medium at the temperature between 12 and 37°C, with the higher activity in the first 7 hours (Fratamico and Cooke, 1996; Fratamico and Whiting, 1995). In disagreement with that evidence, in this experiment, *B. bacteriovorus* preyed on *E. coli* at 4°C. The *E. coli* level also progressively decreased in the control, possibly due to refrigeration. However, *B. bacteriovorus* increased the prey reduction in the test compared to the control throughout the analysis time.

Experiment 2 (A, B)

The aim of this experiment was to evaluate the effect of the milk matrix on the predation of *B. bacteriovorus* against *E. coli* for a time corresponding to the shelf-life of raw milk. Then we tested at 30°C both the boiled milk (2A) and DNB (2B) contaminated with predator/prey

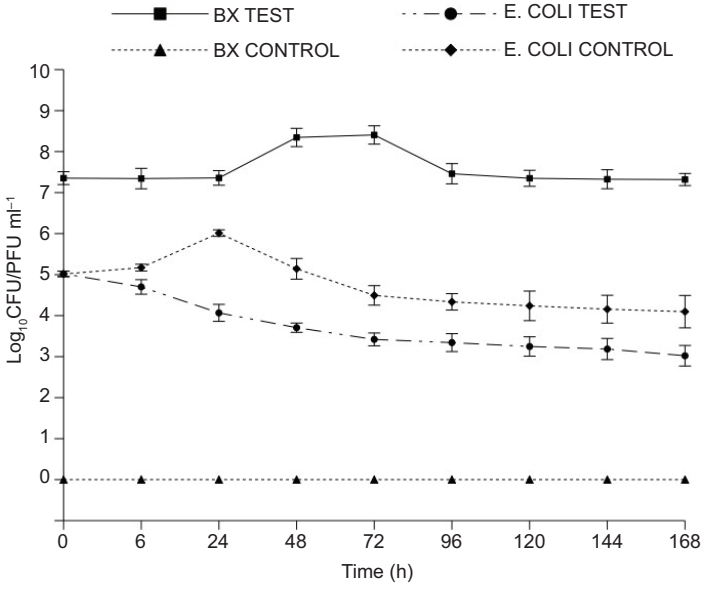

Figure 1. Experiment 1. Growth of *B. bacteriovorus* (BX) and *E. coli* in test (with BX) and control (without BX) boiled milk at 4°C. ♦ *E. coli* control; ● *E. coli* test; ▲ BX control; ■ BX test. Results of microbiological analyses (n = 9) were reported as mean values (Log transformed) ± standard deviation.

ratios to activate the best predation. For both milk and DNB, predator and prey counts were performed at 0, 6, 24, 48, 72 hours from the treatment in test and control. The results are summarized in Figure 2. By comparing predator concentrations at different time points in milk and DNB tests, higher *B. bacteriovorus* levels for all-time points in DNB than in milk were observed, with the greatest significant difference of about 2 Log after 6 hours (t = 3.9497; P = 0.0027). By comparing prey concentrations at different time points in the milk test and control, lower *E. coli* levels were observed in the test than in the control from 6 to 48 hours, with the greatest significant difference by about 3 Log after 6 hours (t = 10.6956; P < 0.0001). By comparing prey concentrations at different time points in milk and DNB tests, lower levels for all-time points in DNB than in milk were observed, with the greatest significant difference of about 4 Log at 48 hours (t = 4.0068; P = 0.0025). *B. bacteriovorus* preyed on *E. coli* more in DNB than in milk. The higher viscosity of milk than in DNB could make the former less suitable for predation than the latter by hindering *B. bacteriovorus* to swim and attack the prey. This hypothesis is reinforced by lower predator counts in milk than DNB for the entire analysis period. Comparing the growth trends of *E. coli* in milk and DNB controls, a lower growth trend was observed in milk than in DNB. This evidence suggests that the milk matrix also slowed prey growth.

Experiment 3 (A, B)

The aim of Experiment 3A was to evaluate the influence of the native milk microbial community on the predation of *B. bacteriovorus* against *E. coli* under the best predation conditions in terms of prey/predator ratio, at the refrigeration temperature for a time corresponding to its shelf-life. Then, we tested at 4°C up to 72 hours, the raw milk contaminated with the predator/prey ratio to obtain the best predation. In Experiment 3B, the growth of *B. bacteriovorus* and its predation toward natural level of indigenous *E. coli* were evaluated in raw milk, under normal storage conditions, for a time corresponding to its shelf-life. Predator and prey counts were performed at 0, 6, 24, 48, 72 hours from the treatment in test and control. The results are summarized in Figure 3. The predator trends in boiled and raw milk (Experiments 1 and 3A) were similar. Lower *E. coli* levels were observed in the test compared to the control for all time points, with the greatest significant difference of about 2 Log after 24 hours (t = 5.9747; P = 0.0001). *B. bacteriovorus* demonstrated a greater (3 Log) and faster (after 6 hours) capacity to contain prey in the milk test at 30°C than at 4°C, that is, 2 Log after 24 hours. This evidence is realistic as 30°C is the optimum temperature for predation. Similar prey concentrations were present in the boiled and raw milk tests (Experiments 1 and 3A), at all the time points. This evidence suggests that when *E. coli* is at an optimal concentration for *B. bacteriovorus* predation, the indigenous microorganisms of milk potentially competing with *E. coli* as prey, do not affect the predator's performance toward its favorite prey. Raw milk represents a relevant source of infection by STECs (Caprioli *et al*., 2015; Jang *et al*., 2017). We tested *B. bacteriovorus* with a non-pathogenic strain of *E. coli*, however, previous work had shown that the growth rates of pathogenic and non-pathogenic *E. coli* are similar (Cassin *et al*., 1998).

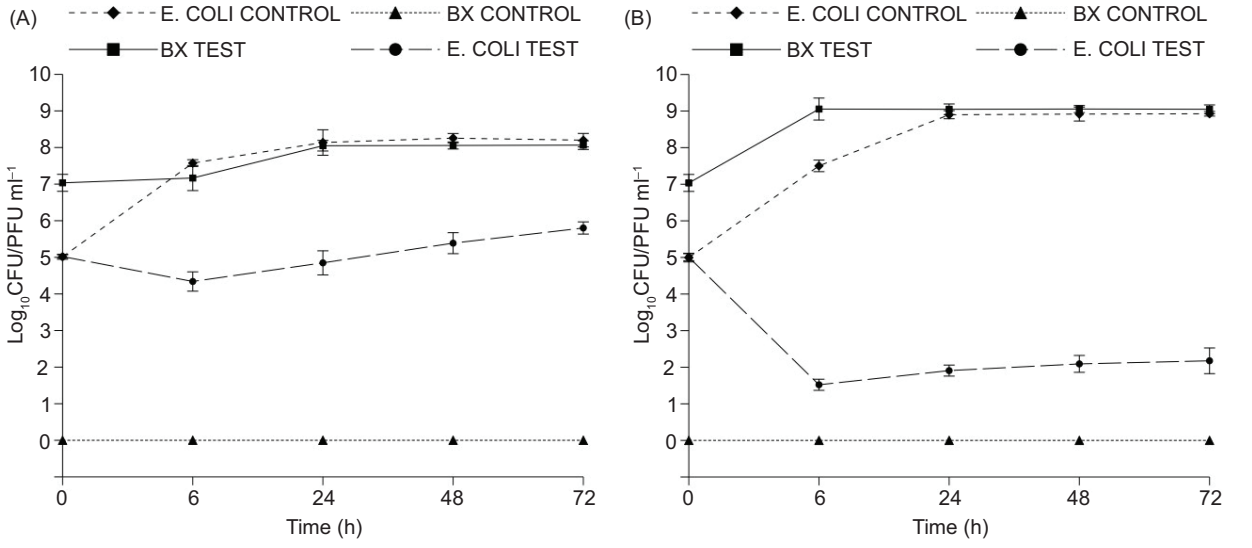

Figure 2. Experiment 2. Growth of *B. bacteriovorus* (BX) and *E. coli* in test (with BX) and control (without BX) boiled milk (A) and DNB (B) at 30°C. ♦ *E. coli* control; ● *E. coli* test; ▲ BX control; ■ BX test. Results of microbiological analyses (n = 9) were reported as mean values (Log transformed) ± standard deviation. DNB, diluted nutrient broth.

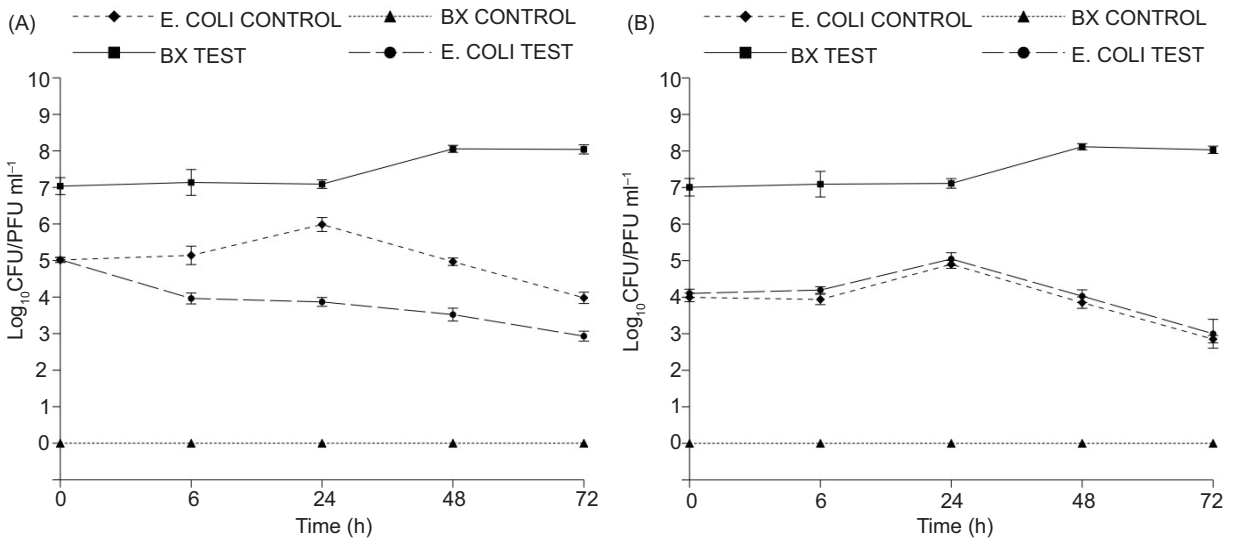

Figure 3. Experiment 3. Growth of *B. bacteriovorus* (BX) and *E. coli* experimentally added in test (with BX) and control (without BX) raw milk at 4°C (A); Growth of *B. bacteriovorus* (BX) and indigenous *E. coli* in test (with BX) and control (without BX) raw milk at 4°C (B) ♦ *E. coli* control; ● *E. coli* test; ▲ BX control; ■ BX test. Results of microbiological analyses (n = 9) were reported as mean values (Log transformed) ± standard deviation.

Moreover, in our previous study, *B. bacteriovorus* showed lytic ability toward STECs and multidrug-resistant *E. coli* strains of different origins (Ottaviani *et al*., 2019). So it is realistic to think that *B. bacteriovorus* could similarly contain STECs if they are in raw milk. In Experiment 3B, the predator trend was like that of Experiment 3A where the prey was experimentally added. In the milk control, predator counts were always not detectable. No significant difference in prey concentration between the test and control was observed. However, in the milk test, *B. bacteriovorus* grew between 24 and 48 hours, suggesting that it preyed on indigenous microorganisms other than *E. coli.* Previously, it has been reported that *B. bacteriovorus* showed lytic ability toward psychrotrophic spoilage bacteria like *Pseudomonas* that are commonly found in raw milk (Dashiff *et al*., 2011; Fratamico and Whiting, 1995; Tremonte *et al*., 2014; Vianna *et al*., 2012). Psychrotrophic bacteria and *Pseudomonas* in the raw milk increase during refrigeration, reaching overtime values higher than 6 Log CFU per mL (Tremonte *et al*., 2019; Vianna *et al*., 2012). In light of this, in Experiment 3B, *B. bacteriovorus* might have preyed on psychrotrophic microorganisms. This aspect would confirm *B. bacteriovorus* as a potential candidate to contain gram-negative pathogenic and spoilage bacteria in raw milk, while preserving those gram-positive bacteria, such as streptococci and lactobacilli, which represent part of the natural flora of milk or used as starters in dairy production. This is a preliminary laboratory-scale study to understand whether *B. bacteriovorus* has the potential as a predator in milk. Our results showed that *B. bacteriovorus* at 4°C survived in milk for the entire analysis time, about 1 week, and preyed for up to 48 hours, although refrigeration and the "matrix of the milk" have limited its performance. At the refrigeration temperature, when *E. coli* was at an optimal concentration for the predation, the presence of other potential preys in the milk did not affect the predatory efficiency of *B. bacteriovorus* toward its preferred prey. On the other hand, when *E. coli* was at a low concentration, the predator grew without predating it, probably by preying on other bacteria present in milk at a high concentration, such as psychrotrophs. Our next goal will be to test the predatory activity of *B. bacteriovorus* against native strains of *E. coli* and *Pseudomonas* isolated from raw milk and tested both in monoculture and in mixtures. This will help us understand the dynamics of *B. bacteriovorus* predation and toward which prey the predator shows the greatest affinity.

Moreover, we are going to test *B. bacteriovorus* predation against native *E. coli* in raw milk samples heavily contaminated by this microorganism, extending the study to sheep and goat milk. Finally, it will be assessed whether *B. bacteriovorus* and its metabolites produced during growth have any effect on the organoleptic characteristics, pH, and nutrients of raw milk during its shelf life. It is known that *B. bacteriovorus* reduces but does not eliminate prey because a balance is established in the growth medium that allows the survival of both the prey, although quantitatively reduced, and the predator (Bratanis *et al*., 2020). In light of this, even if *B. bacteriovorus* cannot be used to eliminate prey microorganisms, it can still reduce their microbial load and thus improve the microbiological quality of raw milk during its storage at 4°C. It would also be interesting to test this predator in integrated biological-chemical-physical approaches to

maximize the inhibitory effect on prey milk microorganisms. For example, an innovative and promising approach used CO_2 during 4°C storage of raw milk to reduce its microbial load (Bratanis *et al*., 2020). Recently, it has been demonstrated that *B. bacteriovorus* is part of the intestinal microbiota of vertebrates, including humans, and plays a key role in maintaining health (Bonfiglio *et al*., 2020a, 2020b; Dwidar *et al*., 2012; Iebba *et al*., 2013). As *Bdellovibrios* are natural residents of the intestinal microbial ecosystem, their functionality and stability should not be affected by the chemical–physical characteristics of the intestinal habitat. Probiotics are defined as live microorganisms that confer a health benefit to the host (EFSA, 2018). Assessment of safety, functionality, and stability are the first points to consider for a microorganism in terms of its use as a probiotic (EFSA, 2018). Many *in vitro* and *in vivo* studies have shown that *Bdellovibrios* meet all these requirements (Bonfiglio *et al*., 2020a, 2020b). As far as our knowledge is concerned, this is the first report on the application of *B. bacteriovorus* as a predator in milk: we believe our results are promising and that *B. bacteriovorus* merits further investigation. This approach to decontamination of raw milk based on *Bdellovibrio* is certainly economically sustainable, non-aggressive as it uses microorganisms which are natural components of the human intestinal flora and that also have potential as probiotics.

Conclusions

Due to its biological properties and predation mode, *B. bacteriovorus* could find application in raw milk used as food or raw material during storage at 4°C to reduce the microbial load of *E. coli* including both spoilage and STEC strains and therefore increase its shelf-life, quality, and healthiness. Furthermore, if we confirm that *B. bacteriovorus* preys on the psychrotrophic microorganisms of raw milk and, consequently, also reduces the production of thermostable enzymes, this could also be effective in increasing the shelf life of UHT, pasteurized milk, and other milk-derived foods.

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Declarations

Conflicts of interest/Competing interests

The authors declare that they have no financial/non-financial conflict of interest. The manuscript does not contain experiments involving human participants and/or animals.

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