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ORIGINAL ARTICLE

Growth potential of *Yersinia enterocolitica* in blue cheese and in blue cheese with probiotic -*Lactobacillus acidophilus* LA-5®

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Abstract In the annual reports of the European Food Safety Authority (EFSA), *Yersinia enterocolitica* (YE) is enumerated as the third most common enteric pathogen responsible for food poisonings. The objective of the paper was to determine the potential for *Yersinia enterocolitica* growth in blue cheese and in blue cheese with a probiotic (*Lactobacillus acidophilus* LA-5®). The experiment was carried out with five different cheese batches. The potential of YE growth was determined at 3, 6, 9, 12 and 15 °C in ten time intervals up to 480 h of storage. The initial contamination level was set at ~10^3 cfu/g YE. The studies demonstrated that at lower temperatures there was a systematic increase in the number of cells throughout the storage period, whereas at higher temperatures stationary and die-off phases were observed. By comparing the increase in the number of YE cells in both cheese varieties, it may be observed that at 6, 9 and 12 °C the number of YE cells was lower in blue cheese with probiotic than in blue cheese at each stage of the experiment although it did not guarantee the microbiological safety of the product.

Keywords *Yersinia enterocolitica* · *Lactobacillus acidophilus* LA-5® · Blue cheese

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Introduction

Fermented dairy products, including cheese, are generally thought to be safe regarding infections with pathogenic bacteria. However, many researchers have indicated the possibility of their contamination during the production process and trade (Bishop and Smukowski 2006). Carrasco et al. (2012), using the data of World Health Organization (WHO), reported that 25 % of all food poisoning cases are caused by cross-contamination resulting from non-compliance with good production and hygienic practices. Most of the studies on the growth of pathogens in cheese concern *Listeria monocytogenes* and many authors explain it by the psychrotrophic nature of these rods and their resistance to numerous environmental factors. Moreover, these bacteria are a criterion of food safety according to the Regulation of the European Commission (EC) No 2073/2005. Less attention is being paid to *Yersinia enterocolitica*, despite it being enumerated (in the annual reports of EFSA) as the third-most common enteric pathogen responsible for food poisonings with dairy products indicated as one of the main sources of intoxication cases. *Yersinia enterocolitica* (YE) are psychrotrophic, Gram-negative rods belonging to the *Enterobacteriaceae* family; these bacteria are resistant to many environmental factors. Although the interest of research into YE peaked in the 1980s, interest in these rods is now reemerging. It mainly results from virulent *Y. enterocolitica* O:8 strains classified in 1B biotype being detected in Europe in recent years; these strains have been for many years isolated only in the USA. In addition, a number of reports on the pathogenicity of 1A serovar that has been so far thought to be non-pathogenic is rising (Batzilla et al. 2011; Zadernowska et al. 2014).

The percentage of products that contain probiotics offered in the portfolio of food manufacturers is increasing. Probiotic bacteria have a beneficial effect on the human body by, among

others, an antibacterial action exerted on pathogenic bacteria. There are many publications on this topic. *Lactobacillus acidophilus* LA-5® is one of the probiotic strains that are commonly used in dairy production, as it is documented that it survives on dairy products and inhibits pathogens (Yilmaztekin et al. 2004; Tabasco et al. 2009; Ziarno et al. 2010).

In the literature, there are a limited number of papers on the growth of YE in dairy products, including those with probiotic bacteria. It is interesting to consider the following question: if a probiotic product becomes contaminated with pathogenic bacteria, will they develop at the same rate as in a product without probiotic bacteria?

The objective of the studies was to determine the growth potential of *Yersinia enterocolitica* in blue cheese produced with lactic acid bacteria and *Penicillium roqueforti* mold with a GRAS (generally recognized as safe) status and in blue cheese with a probiotic that is additionally enriched in *Lactobacillus acidophilus* LA-5® probiotic bacteria.

Materials and methods

Cheese samples

Blue cheese without probiotic and with *L. acidophilus* LA-5® originating from the same manufacturer, was purchased in a grocery straight after delivery and it was immediately transported to a laboratory. The experiment was carried out with five different blue cheese batches and five different batches of blue cheese with probiotic. In order to exclude the presence of YE, 25 g of cheese were sampled, homogenized with buffered phosphate (BBPS) (Merck Poland) and incubated at 10 °C for 10 days. Confirmatory cultures were performed with a surface method on a CIN medium (Merck, Poland) supplemented with cephsoldin-irgasan-novobiocin antibiotics (Merck, Poland).

Bacterial strains

The mixture of reference strains from an ATCC collection (*Y. enterocolitica* 23715, *Y. enterocolitica* 27729) and *Y. enterocolitica* isolated at the Department of Industrial and Food Microbiology (University of Warmia and Mazury in Olsztyn), was used to inoculate cheese. The virulence of the strain was confirmed with a simultaneous detection of virulence-related attachment invasion locus (Ail) and *vir*F genes in accordance with the method presented by Thisted Lambertz and Danielsson-Tham (2005). YE strains were cultured on nutritive broth at 25 °C/24 h. The cultures were diluted and mixed at a 1:1:1 ratio.

Cheese inoculation and storage

Samples of 25 g each were weighed into a sterile bag (Interscience). Cheese samples were contaminated with a previously prepared mixture of YE rods in order to produce contamination at ~10^3 cfu/g YE. Contaminated cheese was stored at 3, 6, 9, 12 and 15 °C. Cultures on a CIN medium (Merck, Poland) supplemented with cephsoldin-irgasan-novobiocin antibiotics (Merck, Poland) were performed immediately after inoculation (0 h) and at the following time intervals: 24, 48, 72, 120, 144, 168, 312, 384, 408 and 480 h. The plates were incubated at 30 °C/24–48 h. If, after that period of time, any growth was not observed, incubation was extended by an additional 24 h.

Statistical analysis

SPSS pocket program for windows (version 16, 2007) was used for the statistical analysis. Values of different parameters were expressed as the mean ± standard error (SE). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine significant differences in the measured attributes at $P < 0.05$.

Results

The microbiological analysis of blue cheese demonstrated a statistically significant increase in the number of cells ($P < 0.05$) at 6, 9, 12 and 15 °C within 24 h of incubation. By contrast, at 3 °C a statistically significant increase was observed only after 48 h (3.12 log). In blue cheese with probiotic, statistically significant differences in the number of cells after 24 h of incubation were seen at 12 and 15 °C. After 48 h of storage, there was a statistically significant increase recorded in blue cheese with probiotic at 9 °C, whereas at 3 and 6 °C such an increase was detected after 72 h (Table 1).

In blue cheese, the number of cells at 3 °C did not change statistically between 48 and 384 h of storage. The final number of rods after 480 h of incubation at this temperature was 4.73 log. At 6 °C, no statistically significant differences were observed between 24 and 72 h with the next statistically significant increase seen in 120 h, which did not change until 168 h. Starting from 312 h till the end of the experiment, no statistically significant differences in the number of cells were detected. At 9 °C, a statistically significant increase produced after 24 h was maintained until 120 h and no statistically significant changes in the number of cells were subsequently recorded (4.99 ± 0.5log – 5.44 ± 0.34log). At 12 °C, a statistically significant difference was detected after 24 h, the next between 48 and 72 h with an increase till 168 h and a drop afterward. At 15 °C, a statistically significant increase was detected until 72 h and the number of cells then decreased till

Table 1 Populations (log_{10} CFU/g)* *Yersinia enterocolitica* in cheeses for different lengths of time

Cheese	Storage Temp	Storage time (hours)									
		0	24	48	72	120	144	168	312	384	408	480
Blue cheese with probiotic	3 °C	1.88 ± [a]0.24A[b]a[c]	2.23 ± 0.35Aa	2.33 ± 0.31Aa	2.96 ± 0.13Ba	3.37 ± 0.17Ca	3.8 ± 0.11Da	3.97 ± 0.03DEa	4.33 ± 0.27Ea	4.6 ± 0.12Ea	4.62 ± 0.19Ea	4.73 ± 0.21Ea
	6 °C	1.88 ± 0.87Aa	2.16 ± 0.14Aa	2.34 ± 0.25Aa	2.48 ± 0.39ABac	3.07 ± 0.13Bab	3.55 ± 0.34BCa	3.89 ± 0.14Ca	4.47 ± 0.45CDa	4.48 ± 0.19Da	4.62 ± 0.1Da	4.69 ± 0.12Da
	9 °C	1.89 ± 0.42Aa	2.1 ± 0.13Aa	3.25 ± 0.2Bb	3.39 ± 0.12Bb	3.59 ± 0.2Ba	3.77 ± 0.06Ca	3.97 ± 0.08Da	4.39 ± 0.19Ea	4.33 ± 0.21DEa	4.45 ± 0.11Ea	4.52 ± 0.02Ea
	12 °C	1.90 ± 0.12Aa	2.16 ± 0.24ABa	2.39 ± 0.12Ba	2.37 ± 0.09Bc	2.83 ± 0.22Cb	3.24 ± 0.37CDa	3.72 ± 0.3Da	3.85 ± 0.11Db	4.17 ± 0.24DEa	4.29 ± 0.17DEa	4.6 ± 0.28Ea
	15 °C	1.96 ± 0.37Aa	3.27 ± 0.28Bb	3.6 ± 0.09Bb	4.09 ± 0.17BCb	4.4 ± 0.58BCa	4.23 ± 0.32BCa	4.16 ± 0.19BCa	4.53 ± 0.33BCab	4.57 ± 0.19Ca	4.68 ± 0.25Ca	4.78 ± 0.3Ca
Blue cheese	3 °C	2.37 ± 0.3Aa	2.8 ± 0.6Aa	3.12 ± 0.31ABa	3.31 ± 0.59ABa	3.59 ± 0.79ABa	3.66 ± 0.73ABa	4 ± 0.44Ba	4.24 ± 0.25Ba	4.34 ± 0.28Ba	4.93 ± 0.12Ca	4.98 ± 0.2Ca
	6 °C	2.37 ± 0.37Aa	2.86 ± 0.29ABa	3.31 ± 0.38Ba	3.96 ± 0.4BCa	4.55 ± 0.2Ca	4.99 ± 0.3CDab	5.27 ± 0.47CDb	5.44 ± 0.44Db	5.61 ± 0.48Db	5.71 ± 0.35Db	5.72 ± 0.61Db
	9 °C	2.38 ± 0.27Aa	2.94 ± 0.24ABa	3.9 ± 0.55Bab	4.26 ± 0.45Bab	4.99 ± 0.5BCab	5.41 ± 0.21Cb	5.54 ± 0.26Cb	5.51 ± 0.27Cb	5.6 ± 0.23Cb	5.58 ± 0.23Cb	5.44 ± 0.34Cb
	12 °C	2.39 ± 0.37Aa	3.11 ± 0.15Ba	4.39 ± 0.34Cb	5.04 ± 0.34CDb	5.43 ± 0.15Db	6.02 ± 0.27Ebc	6.04 ± 0.27Ebc	6.02 ± 0.36DEb	5.65 ± 0.22DEb	5.31 ± 0.3DEb	5.18 ± 0.4CDEb
	15 °C	2.43 ± 0.4Aa	4.19 ± 0.17Bb	5.59 ± 0.32BCc	5.96 ± 0.22BCc	6.2 ± 0.29Cc	6.18 ± 0.29BCc	6.34 ± 0.22BCc	6.03 ± 0.41Cb	5.72 ± 0.32Cb	5.84 ± 0.2Cc	5.39 ± 0.31Cb

[a] Data represent mean ± standard deviation of 5 measurements

[b] Means with the same capital letter within a row are not significantly different ($P > 0.05$)

[c] Means with the same lowercase letter within a column for the same type of cheese are not significantly different ($P > 0.05$)

312 h, although this reduction was statistically insignificant (Table 1).

In blue cheese with probiotic, at 3 °C there was a statistically significant increase between 72 and 144 h and the number of cells then increased from 168 h, although statistically insignificantly. At 6 °C, no statistical differences were detected between 24 and 72 h; the next statistically significant increase was recorded after 120 h and did not change statistically significantly until 144 h. Afterward, the number of cells increased statistically, yet after 312 h no statistically significant differences were recorded. At 9 °C, a statistically significant increase was detected after 48 h and it lasted till 120 h. The next increase was recorded after 144 and 168 h, with no statistically significant changes in the number of cells recorded thereafter (4.39 ± 0.19log – 4.52 ± 0.02log). At 12 °C, a statistically significant difference was recorded after 24 h and the next after 120 h while between 168 and 408 h no statistically significant changes were observed. Afterward, there was an increase in the number of cells till the end of the experiment, although it was statistically insignificant. At 15 °C, from 72 h until the end of the experiment there was a continuous, yet statistically insignificant, increase (Table 1).

Comparing the increase in the number of YE cells in both cheese varieties, it is seen that at 6, 9 and 12 °C the number of YE cells was lower in blue cheese with probiotic than in blue cheese at each stage of the study. These differences were significantly lower at 15 °C, while they were not at all reported at 3 °C (Fig. 1).

Discussion

Blue cheese is a very popular cheese variety that is consumed as a snack as well as a component of salads and sandwiches. It is most often delivered to groceries in blocks weighing several kilograms that are portioned individually for clients, which may favor cross-contamination. Cheese is most commonly stored under refrigerating conditions, which limits the growth of other *Enterobacteriaceae*, yet facilitates the multiplication of psychrotrophs (including YE) that consequently dominate the environment. As indicated in studies by Divya and Varadaraj (2013), although YE is not heat-resistant and should not survive the whole cheese production process, it is still possible in some cases. The so-called "food matrix", namely, its composition, processing temperature, phase of bacterial growth, specific strain properties, etc., has a substantial impact.

Both our experiment and studies by other authors demonstrated the potential for the survival and growth of YE in dairy products. Gulmez and Guven (2003) showed that YE might grow during fermentation of kefir and its storage up to 336 h. Hanifian and Khani (2012) conducted studies on Lighvan cheese that was contaminated during production with

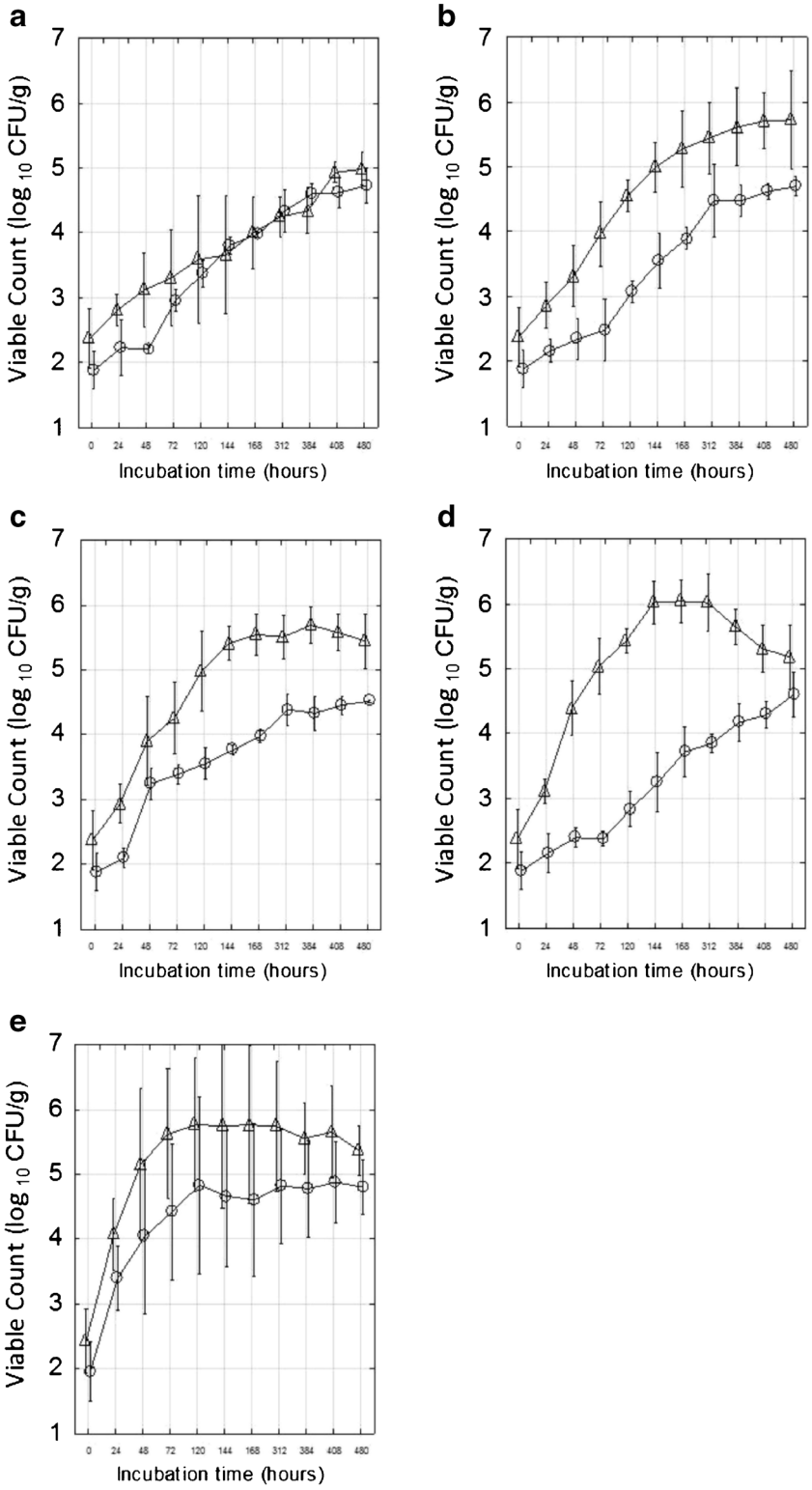

Fig. 1 Grow of Y. *enterocolitica* during storage at 3 °C (**a**), 6 °C (**b**), 9 °C (**c**), 12 °C (**d**) and 15 °C (**e**) in Blue cheese (Δ), Blue cheese with probiotic (○)

three YE strains at 3log cfu/g. After 720 h of storage at 4 °C, live YE cells were still detected at 1 log cfu/g. Lazarte Otero et al. (2010) contaminated samples of goat cheese with YE at 1×10^6 cfu/g and stored them at 4 and 22 °C for 120 days. Viable YE cells were recorded at a high level (over 4log cfu/g) until 168 h at 22 °C and till 720 h at 4 °C. Both our experiment and studies by other authors clearly indicate that due to the psychrotrophic nature of YE, storage temperature exerts a significant impact on the growth and survival of this microorganism in dairy products. Our studies demonstrated that at

lower temperatures there was a systematic increase in the number of cells throughout the storage period, while at higher temperatures, stationary and die-off phases were observed. At 15 °C, there were significant statistical deviations between the results. Blue cheese is a medium for the growth of different microbial groups; at 15 °C there is usually intensive growth and complex inter-species interactions, which may be reflected in differences between the results of individual samples (Fig. 1). Furthermore, our studies were conducted on cheese of five different batches and thus they may have slightly differed in composition, which significantly impacted the large statistical differences in YE growth given that the cheese was contaminated with three YE strains.

An important stage of our studies was an attempt at answering the question of whether YE would grow at the same rate in blue cheese and in blue cheese with probiotic bacteria. Data in the literature indicates the potential for inhibition of pathogenic bacterial growth by probiotic bacteria. El-Kholy et al. (2014) conducted studies on the impact of different probiotic bacteria on pathogens in yoghurt and found that *L. acidophilus* La-5 showed the highest inhibitory properties. These authors, however, did not study the interactions between *L. acidophilus* La-5 and YE. Unfortunately, most studies on the potential for inhibition of pathogens in dairy products by probiotic strains have focused on investigating their impact on the growth of *Salmonella* sp., *Listeria monocytogenes* and *Staphylococcus aureus*. Mahmoudi et al. (2012) conducted studies on the impact of *Lactobacillus acidophilus* probiotic bacteria on the growth of *Listeria monocytogenes* in white cheese. The results thereof, similar to our findings, indicate less intensive growth of the examined pathogens in a product with probiotic bacteria. The addition of probiotics to cheese may prolong its shelf-life and improve its microbiological safety by delaying the growth of bacteria that are typical contaminants and pathogenic bacteria (Madureira et al. 2011).

Conclusions

Blue cheese may be an environment for YE growth. Considering that these cheese varieties have a relatively long shelf-life and the fact that YE grows systematically under refrigerating conditions in the case of contamination, they may present a threat to human health. As demonstrated by our studies, the increase of YE in cheese with *Lactobacillus acidophilus* La-5 culture was less intensive than in cheese without this additive yet this did not guarantee the safety of the product.

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