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EFFECT OF INCREASING ZEARALENONE LEVELS ON THE TECHNOLOGICALLY PROBLEMATIC MICROORGANISMS AND FOOD RISKY PATHOGENS (IN VITRO)

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Abstract

The aim of this study was to determine the effect of different zearalenone (ZEA) concentrations (0, 10, 100, 250, 500, 1 000 µg/l) on growth of *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas fluorescens* in milk. The samples were incubated for 6 days at 22 and 30 °C (*B. cereus* and *S. aureus*) or at 6.5 and 22 °C (*P. fluorescens*), respectively. Counts of bacteria in milk were measured every 24 hours. Maximum counts of *B. cereus* after 144 h of incubation at 30 °C ranged from 7.00 to 7.78 log CFU/ml. The most significant effect of ZEA on *B. cereus* across the experiment was observed after 96 h of incubation at 22 °C and after 72 h at 30 °C ($P < 0.001$ and $P < 0.01$, respectively). *S. aureus* maintained at 30 °C showed similar growth parameters as at 22 °C regardless of ZEA presence. The most significant effects of ZEA on *S. aureus* were after 120 h of incubation at 22 °C and after 72 h at 30 °C ($P < 0.001$). The growth of *P. fluorescence* at 6.5 °C was slower compared to growth at 22 °C. The most significant effect of ZEA between ZEA spiked and Z0 samples on *P. fluorescence* across the experiment was observed after 48 hours of incubation at 22 °C ($P < 0.001$).

Keywords: mycotoxin, milk, viability, *Bacillus cereus, Staphylococcus aureus, Pseudomonas fluorescens*

INTRODUCTION

Because milk and dairy products are significant components of human diet great emphasis is placed on their safety. Although the sources of milk contamination are multiple, microbiological hazards are a major food safety concern in the dairy sector. Microbes that may be present in milk can include not only conditionally beneficial microorganisms such as lactic acid bacteria but also microorganisms linked to detrimental effects on human health (pathogens) or product quality (spoilage microorganisms) (Boor *et al*., 2017).

As a foodborne pathogen *Staphylococcus aureus* (*S. aureus*, Gram-positive coccal bacterium) is responsible for a variety of health problems and diseases related to food consumption (Jamali *et al*., 2015). *S. aureus* belongs to the most frequent microbial contaminant of raw milk and its occurrence depends on the type of milk and geographical origin (Valík *et al*., 2018). Its prevalence in bovine milk ranges from 47 to 75% (Jørgensen *et al*., 2005; Mehli *et al*., 2017).

Bacillus cereus (*B. cereus*, Gram-positive aerobic spore-forming bacterium) is one of the most significant spoilage microorganisms found in milk and dairy products (Vyletělová *et al.,* 2000) because the spores are heat-resistant and can survive thermal treatments of milk, grow during storage temperatures (Páčová *et al.,* 2003) and germinate rapidly upon heat activation (Noriega *et al.,* 2003). As a producer of various extracellular enzymes *B.cereus* can be responsible for spoilage and decreased organoleptic quality of milk (Kumari and Sarkar, 2016). Furthermore, it may also cause foodborne diseases due to the production of toxins (Andersson *et al.*, 1995).

Pseudomonas spp., particularly *Pseudomonas fluorescens* (*P. fluorescens*, Gram-negative psychrotrophic bacteria) are the most commonly isolated bacteria from raw milk (Vyletělová and Hanuš, 2000) with a significant spoilage potential due to production of lipolytic and proteolytic enzymes during the storage at lower temperatures, which decreases quality and shelf life of milk and dairy products (Vyletělová *et al.,* 2000). Moreover, *P. fluorescens* is highly heterogeneous species and includes virulent and sub-clinical strains that are involved in opportunistic nosocomial infections (Rossignol *et al.*, 2009).

The other possible way of raw milk contamination is via dietary mycotoxins with proved carry-over from feed into milk (Becker-Algeri *et al.,* 2016; Flores-Flores *et al.,* 2015). Zearalenone (ZEA) is one of the main mycotoxin contaminants of dairy diets based on maize silages (Becker-Algeri *et al.,* 2016) and recently its occurrence in milk and dairy products was reported as summarised in our previous study (Hanuš *et al.,* 2018). Except of health effects on human and animals, such as estrogenic activity, hepatotoxicity, hematotoxicity or imunotoxicity (Tinyiro *et al.,* 2011), effects of ZEA on growth and viability of cells were reported (Zhang *et al.,* 2020; Hanuš *et al.,* 2018; Zheng *et al.,* 2018). However, the interaction between ZEA and above mentioned milk pathogens and spoilage bacteria was not studied. Thus, the aim of the study was to evaluate the effect of increasing levels of ZEA on growth of selected contaminant microorganisms including pathogenic at different temperatures.

MATERIALS AND METHODS

Selection and Preparation of Strains

A strain of *B. cereus* (26 B, own collection; isolated from raw cow milk) and a reference strain of *S. aureus* (Czech Collection of Microorganisms, Masaryk University, Brno; CCM 6188) were multiplied in a broth (10 g Pepton, 10 g Lab Lemco Poder, 5 g NaCl, 1000 ml distilled water) at 36 °C for 24 h. Two strains of *P. fluorescens,* ZB 66 (own collection; isolated from raw cow milk) and CCM 2826 (reference strain) were multiplied in a *Pseudomonas* broth containing 1% of glycerol and 0.1% of milk powder (Merck) at 30 °C for 24 h. Then the counts of bacteria (CFU/ml) were determined (ČSN EN ISO 7218, 2008).

Preparation, Inoculation and Incubation of Milk Samples

As an experimental medium the UHT milk (1.5% of fat) was chosen. Prior the experiment counts of mesophilic and spore-forming bacteria were determined with negative results. The 100 ml samples were inoculated with multiplied cultures of *B. cereus* and *S. aureus*. Starting counts of *B. cereus* were 75 and 72 CFU/ml for temperature of 22 °C and 67 CFU/ml for temperature of 30 °C. Counts of *S. aureus* were 86 and 142 CFU/ml for 22 °C and 86 CFU/ml for 30 °C, respectively. Starting concentrations of *P. fluorescens* were 480 CFU/ml (strain ZB 66) and 585 CFU/ml (strain CCM 2826) for both temperatures of 22 °C and 6.5 °C. Individual milk samples were spiked with ZEA (purity min. 98%, purchased from Sigma-Aldrich, Germany) solution diluted in ethanol (Hanuš *et al.,* 2018) to reach the concentrations of 10, 100, 250, 500 and 1000 µg/l, it is groups Z10, Z100, Z250, Z500 and Z1000, respectively. Two controls were used in the experiment:

1. control growth of microorganisms without ZEA and ethanol (C);
2. control growth of microorganisms with ethanol and without ZEA (Z0).

Samples were cultivated for 6 days and sampled after 24, 48, 72, 96, 120 and 144 h of incubations according to the applicable standard for the cultivation of given micro-organism, (*B. cereus*) (ČSN ISO 7932, 1995), (*S. aureus*) (ČSN EN ISO 6888–1, 1999) and (*P. fluorescens*) (ČSN ISO 8552, 2005). The results of *B. cereus* and *S. aureus* were expressed as the average of four measurements for each species while results of *P. fluorescens* were expressed as the average of four values (2 values of *P. fluorescens* ZB 66 and 2 values of *P. fluorescens* CCM 2826) (ČSN EN ISO 7218, 2008).

Calculations and Statistics

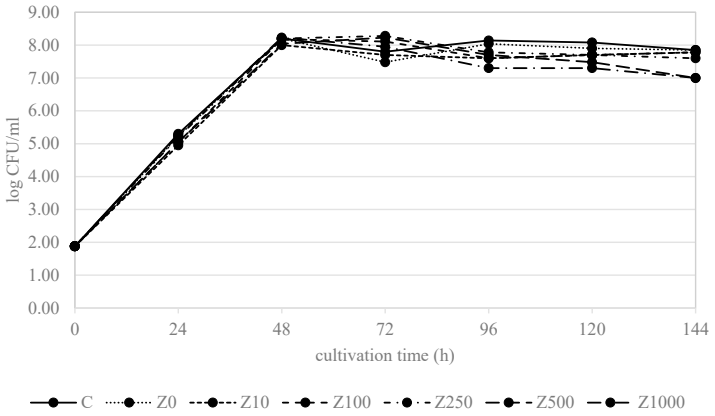
The obtained microbiological values were transformed logarithmically for better comparison of growth curves among the tested microorganisms (Hanuš *et al.,* 2011). The basic statistical characteristics were calculated using the Microsoft Office Excel 2003 (Redmond, Washington, USA). A paired *t*-test was performed to demonstrate the significance of the differences in results between the selected segments of the growth curves of the microorganisms without influence and under the influence of the ZEA addition.

RESULTS

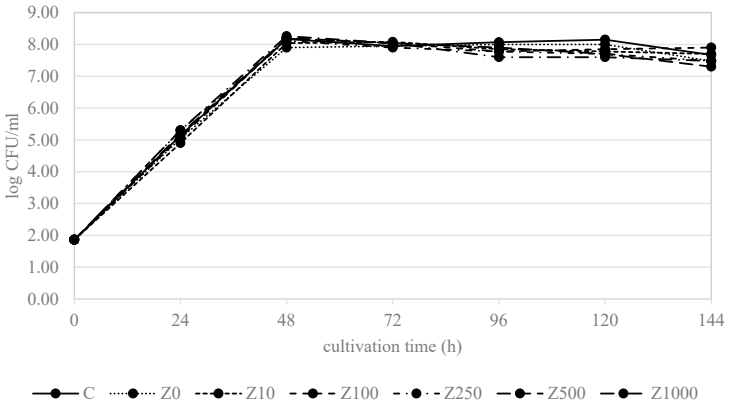
Bacillus cereus

The growth curves of *B. cereus* are shown in Fig. 1 to 3. From these values it is evident no reduction in growth of *B. cereus* during 144 h of

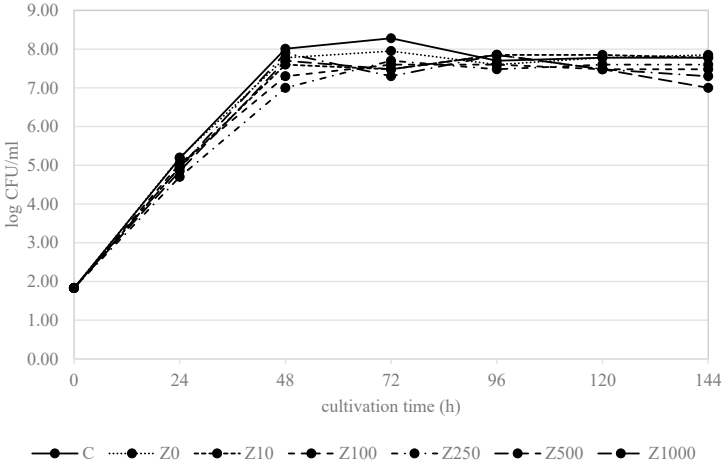
incubation at both 22 and 30 °C even when high concentrations of ZEA were used. In general, the counts of *B. cereus* were rising sharply and reached cca 8 log CFU/ml within 48 h of incubation regardless of initial inoculation level, cultivation temperature or ZEA concentration. At the end of incubation at 22 °C, the total bacterial counts ranged from 7.00 to 7.85 log CFU/ml (initial inoculation of 75 CFU/ml). Similar counts of *B. cereus* ranging from 7.30 log CFU/ml (Z500) to 7.90 log CFU/ml (Z100) were observed also in a case of initial inoculation of 72 CFU/ml. After 144 h of incubation at 30 °C, the



1: *Growth curves of Bacillus cereus (inoculated at 75 CFU/ml) in zearalenone–spiked milk at 22 °C*



2: *Growth curves of Bacillus cereus (inoculated at 72 CFU/ml) in zearalenone–spiked milk at 22 °C*



3: *Growth curves of Bacillus cereus (inoculated at 67 CFU/ml) in zearalenone–spiked milk at 30 °C*

I: *Results of differences and their significance between selected segments of the growth curves of Bacillus cereus without and with zearalenone addition (n = 5)*

Cultivation temp. (°C)	Cultivation time (h)	d ± sd	*t*	P	d ± sd (log)	*t*	P
22[1]	24	0.40 ± 0.386	2.07	ns	0.13 ± 0.128	2.03	ns
	48	0.20 ± 0.309	1.29	ns	0.07 ± 0.103	1.36	ns
	72	-0.95 ± 0.577	3.29	*	-0.57 ± 0.235	4.85	**
	96	0.68 ± 0.148	9.19	***	0.44 ± 0.182	4.84	**
	120	0.39 ± 0.141	5.53	**	0.32 ± 0.181	3.54	*
	144	0.35 ± 0.251	2.79	*	0.42 ± 0.401	2.09	ns
22[2]	24	-0.40 ± 0.523	1.53	ns	-0.11 ± 0.168	1.31	ns
	48	-0.67 ± 0.276	4.86	**	-0.26 ± 0.085	6.12	**
	72	-0.17 ± 0.156	2.18	ns	-0.07 ± 0.069	2.03	ns
	96	0.38 ± 0.147	5.17	**	0.22 ± 0.114	3.86	*
	120	0.46 ± 0.118	7.80	**	0.27 ± 0.094	5.74	**
	144	-0.12 ± 0.235	1.02	ns	-0.09 ± 0.232	0.78	ns
30[3]	24	0.77 ± 0.225	6.84	**	0.20 ± 0.270	4.65	**
	48	0.20 ± 0.270	1.48	ns	0.28 ± 0.354	1.58	ns
	72	0.55 ± 0.114	9.65	***	0.44 ± 0.150	5.87	**
	96	-0.17 ± 0.200	1.70	ns	-0.13 ± 0.175	1.49	ns
	120	0.20 ± 0.178	2.25	ns	0.20 ± 0.161	2.48	ns
	144	0.39 ± 0.192	4.06	*	0.42 ± 0.298	2.82	*

[1] = inoculated at 75 CFU/ml, [2] = inoculated at 72 CFU/ml, [3] = inoculated at 67 CFU/ml
d = mean difference; sd = standard deviation of difference; *t* = value of paired *t*-test criterion; P = significance; ns = non-significant ($P > 0.05$); * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$.

counts of *B. cereus* were from 7.30 to 7.78 log CFU/ml. The statistical significance of differences between samples with (Z10–Z1000) and without zearalenon (Z0) have also not been unequivocally demonstrated even the most significant influences ($P < 0.001$ and $P < 0.01$) were observed after 96 hours of incubation at 22 °C and after 72 hours at 30 °C (Tab. I).

Staphylococcus aureus

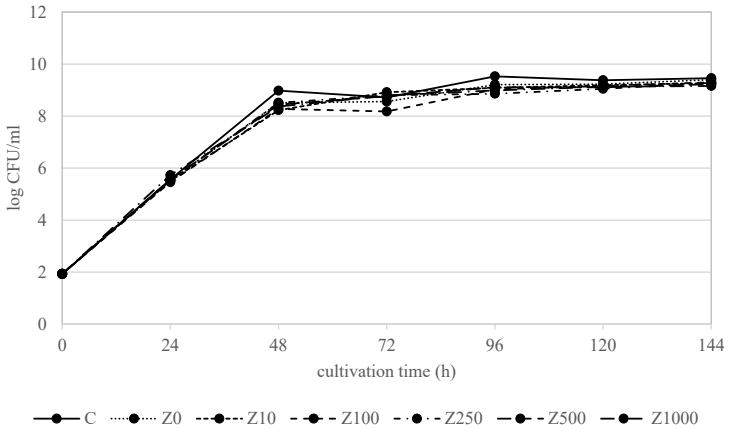
The growth curves of *S. aureus* are shown in Fig. 4 to 6. In general, we can state, that ZEA didn't have significant effect on growth of *S. aureus* under the conditions described above. As expected *S. aureus* after initial inoculation of 86 CFU/ml maintained at 30 °C showed similar growth parameters and reached similar final values as at temperature 22 °C regardless of ZEA presence. The maximum count of *S. aureus* after 144 h of incubation at 30 °C ranged between 9.08 (Z250) and 9.79 (C) log CFU/ml. The similar results have been achieved for the samples with initial concentration 142 CFU/ml. The statistical significance of differences between samples with (Z10–Z1000) and without zearalenon (Z0) have also not been again unequivocally demonstrated. The most significant influences ($P < 0.001$) were observed after 120 hours of incubation at 22 °C and after 72 hours at 30 °C (Tab. II).

Pseudomonas fluorescens

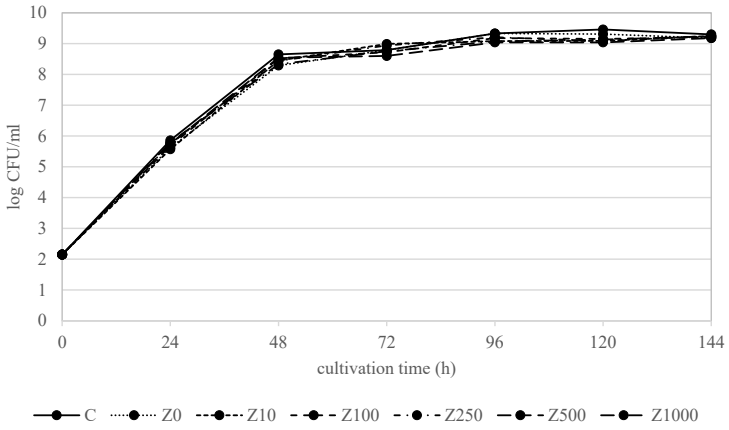
The growth curves of *P. fluorescens* for both temperatures are shown in Fig. 7 and 8. The growth of *P. fluorescence* at 6.5 °C, was slower compared to growth at 22 °C resulting in the highest count of 7.88 log CFU/ml in C after 144 h of incubation. The growth curves of *P. fluorescence* at 22 °C were complete with rapid growth during first 24 h and stationary phase during the remaining part of the experiment. The final counts of bacteria at the end of incubations were similar and no effect of ZEA on growth of *P. fluorescence* was observed. The statistical significance of differences between samples with (Z10–Z1000) and without zearalenon (Z0) has not been also in this case unequivocally demonstrated. The most significant influences ($P < 0.001$) were observed after 48 hours of incubation at 22 °C (Tab. III).

DISCUSSION

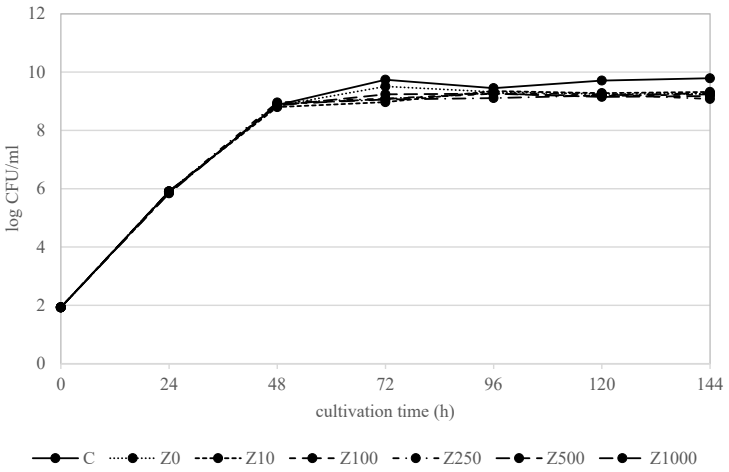
As mentioned in our previous study (Hanuš *et al.*, 2018) the concentrations of ZEA used in this study reflected concentrations of ZEA found in milk (Z10) or maximum levels in foodstuffs (Z100 and Z250) or their multiples (Z500 and Z1000).



4: *Growth curves of Staphylococcus aureus (inoculated at 86 CFU/ml) at different zearalenone concentration at 22 °C*



5: *Growth curves of Staphylococcus aureus (inoculated at 142 CFU/ml) at different zearalenone concentration at 22 °C*

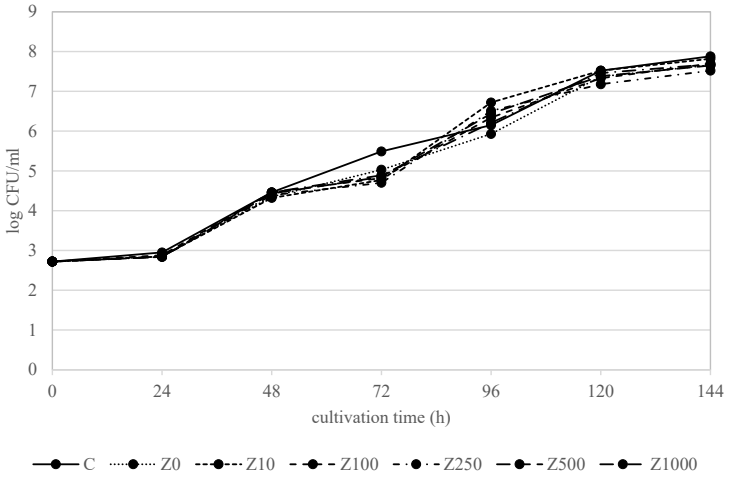


6: *Growth curves of Staphylococcus aureus (inoculated at 86 CFU/ml) at different zearalenone concentration at 30 °C*

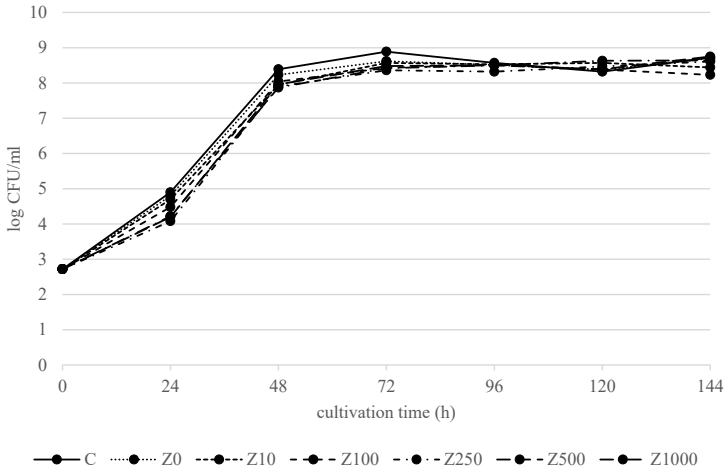
II: *Results of differences and their significance between selected segments of the growth curves of Staphylococcus aureus without and with zearalenone addition (n = 5)*

Cultivation temp. (°C)	Cultivation time (h)	d ± sd	*t*	P	d ± sd (log)	*t*	P
22[1]	24	-0.51 ± 0.963	1.06	ns	-0.05 ± 0.102	0.98	ns
	48	0.87 ± 0.613	2.84	*	0.14 ± 0.114	2.46	ns
	72	-2.00 ± 2.493	1.60	ns	-0.13 ± 0.292	0.89	ns
	96	0.57 ± 0.223	5.11	**	0.19 ± 0.099	3.84	*
	120	0.36 ± 0.164	4.39	*	0.10 ± 0.053	3.77	*
	144	0.72 ± 0.215	6.70	**	0.15 ± 0.054	5.56	**
22[2]	24	-1.00 ± 0.951	2.10	ns	-0.09 ± 0.086	2.09	ns
	48	-0.94 ± 0.512	3.67	*	-0.17 ± 0.083	4.10	*
	72	-0.52 ± 2.490	0.42	ns	-0.01 ± 0.162	0.12	ns
	96	0.84 ± 0.218	7.71	**	0.21 ± 0.074	5.68	**
	120	0.79 ± 0.127	12.44	***	0.21 ± 0.043	9.77	***
	144	-0.17 ± 0.099	3.43	*	-0.04 ± 0.029	2.76	ns
30[3]	24	0.05 ± 0.604	1.66	ns	0.02 ± 0.034	1.18	ns
	48	-0.65 ± 1.048	1.24	ns	-0.03 ± 0.060	1.00	ns
	72	2.00 ± 0.292	13.70	***	0.42 ± 0.097	8.66	***
	96	0.26 ± 0.346	1.50	ns	0.07 ± 0.088	1.59	ns
	120	0.16 ± 0.196	1.63	ns	0.04 ± 0.051	1.57	ns
	144	0.13 ± 0.350	0.74	ns	0.04 ± 0.094	0.85	ns

[1] = inoculated at 86 CFU/ml, [2] = inoculated at 142 CFU/ml, [3] = inoculated at 86 CFU/ml
d = mean difference; sd = standard deviation of difference; *t* = value of paired *t*-test criterion; P = significance; *ns* = non-significant ($P > 0.05$); * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$



7: *Growth curves of Pseudomonas fluorescens ZB 66 and CCM 2826 at different zearalenone concentration (Z) at 6.5 °C (average of 4 log CFU/ml)*



8: *Growth curves of Pseudomonas fluorescens ZB 66 and CCM 2826 at different zearalenone concentration (Z) at 22 °C*

III: *Results of differences and their significance between selected segments of the growth curves of Pseudomonas fluorescens ZB 66 and CCM 2826 without and with zearalenone addition (n = 5)*

Cultivation temp. (°C)	Cultivation time (h)	d ± sd	*t*	P	d ± sd (log)	*t*	P
6.5	24	-0.03 ± 0.290	0.21	ns	0.00 ± 0.017	0.00	ns
	48	-0.35 ± 0.334	2.10	ns	-0.06 ± 0.059	2.03	ns
	72	0.41 ± 0.107	7.66	**	0.22 ± 0.074	5.95	**
	96	-2.16 ± 1.391	3.11	*	-0.51 ± 0.191	5.34	**
	120	-0.09 ± 0.689	0.26	ns	0.00 ± 0.131	0.00	ns
	144	-0.23 ± 1.181	0.39	ns	-0.01 ± 0.108	0.19	ns
22	24	3.79 ± 1.571	4.82	**	0.46 ± 0.250	3.68	*
	48	0.80 ± 0.133	12.03	***	0.29 ± 0.065	8.92	***
	72	1.32 ± 0.535	4.93	**	0.17 ± 0.079	4.30	*
	96	0.19 ± 0.542	0.70	ns	0.04 ± 0.087	0.92	ns
	120	-0.78 ± 0.826	1.89	ns	-0.11 ± 0.110	2.00	ns
	144	1.35 ± 1.511	1.79	ns	0.17 ± 0.203	1.67	ns

d = mean difference; sd = standard deviation of difference; *t* = value of paired *t*-test criterion; P = significance; ns = non-significant ($P > 0.05$); * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

Growth of Bacteria

Bacillus cereus is a bacterium which can/may occur in raw milk (Vyletělová *et al.,* 2002). *B. cereus* grows at temperature range from 4 to 50 °C and the optimum for growing is from 25 to 37 °C (Bhunia, 2008). Maximal growth (100%) of *B. cereus* was observed at 30 °C for 48 hours in raw milk (Montanhini *et al.* 2013) and multiplication of *B. cereus* in pasteurized milk at 8, 15 and 22 °C (Necidová *et al.,* 2014). Our results confirmed the growth and multiplication at 22 °C and also at 30 °C in accordance with above mentioned studies. On the other hand, faster growth of *B. cereus* in pasteurized milk at 30 °C with maximum reached after 8 hours of incubation described Wong *et al.,* (1988). The final counts of *P. fluorescens* after 144 h incubation at 6.5 °C and after 48 h at 22 °C were lower than that reported by Colantuono *et al.* (2020) for 4 °C and 25 °C but close to values found by Miguel *et al.* (2019) for incubations at 7 °C for 6 days. Discrepancies in the growth parameters of bacteria can be explained by a different composition of culture medium or incubation conditions.

Effect of Zearalenone on Milk Pathogens

Generally, the interactions between mycotoxins and bacteria are studied mainly from two reasons – for the ability of some microorganisms to detoxify mycotoxins and for the ability of mycotoxins to change, either quantitatively or qualitatively, composition of the intestinal microbiota when animals are exposed to dietary mycotoxins. Concerning to

ZEA-degrading abilities, several species of *Lactobacillus, Pseudomonas, Bacillus, Lysinibacillus, Rhodoccous* or *Streptomyces* have been identified to degrade ZEA (Venkatesh, Keller, 2019; Cheng *et al.,* 2016). On the other hand, information about the effect of ZEA on viability of microorganisms are scarce and inconsistent. In the present study mild negative effect of ZEA on growth of studied bacteria was observed but it differed in dependence on the incubation time and temperature and the type of bacteria (see Tabs. I, II, III). This is in agreement with the results of our previous study (Hanuš *et al.,* 2018) but in discrepancy with Vyletělová *et al.* (2005) who described no significant difference in the growth of yoghurt culture YC–180–YO–Flex containing *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. lactis* and *L. delbrueckii subsp. bulgaricus* strains when incubated with graded concentration of ZEA. This discrepancy can be explained by different mechanisms of ZEA toxicity as affected by different doses and cell types because high doses of ZEA can cause cell death, while low doses can stimulate the proliferation of cells (Zheng *et al.,* 2018). Concerning to cell type, ZEA may exert the stimulating effects in the cells from tissues with estrogen receptors but the cytotoxic effects in the cells from the tissues which have no estrogen receptors (Zheng *et al.,* 2018). Furthermore, length of exposure may also play a role in the ZEA effects on cells because Piotrowska *et al.* (2014) noted a detrimental effect of a long-term exposure to low levels of ZEA on aerobic mesophilic bacteria in the pig's colon while Wang *et al.* (2018) found no effect of short-term ZEA exposure on the predominant bacteria of the jejunum. According to Zhang *et al.* (2020) ZEA treatment slightly increased the intestinal microbiota diversity but significantly decreased the β diversity. Concerning to microbiota structure, exposure to ZEA caused decrease in the abundance of *Firmicutes* and increase in the abundace of *Bacteroidetes*. Further studies are needed to clarify factors influencing the effect of ZEA on growth and viability of microorganisms.

CONCLUSION

The results mentioned above didn't fully correspond to the effect of ZEA on milk cultures. Selected microorganisms of *B. cereus*, *S. aureus* and *P. fluorescens* were only sporadically reduced in their growth by the mycotoxin ZEA in dependence on the incubation time and temperature, the type of bacteria and initial inoculation counts. The susceptibility of selected natural and pathogenic dairy microorganisms to the inhibitory effect of ZEA has thus been proven to a small extent.

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