Quantification of *Geotrichum candidum* Growth in Co-Culture with Lactic Acid Bacteria

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Abstract: The growth dynamics of filamentous fungus *G. candidum* was studied during the co-cultivation with the commercial lactic acid bacteria (LAB) culture Fresco. The experiments were carried out in milk and on the surface of a milk agar at the temperature ranging from 5 to 37°C. Ratkowsky model was used to describe the relationships of the fungal growth rate to the temperature during both, single and co-cultivation with LAB in milk. Simultaneous growth of LAB affected significantly the growth rate of the filamentous fungus. The growth of *G. candidum* was in average 39% slower in the co-culture than in the single cultivation. LAB pre-inoculated and growing in the solid medium did not show any significant inhibitory effect on the surface growth of *G. candidum* at all tested temperature. The precise data describing the growth of this cheese yeast-like fungus, *G. candidum,* may fill a gap in the field of quantitative food mycology and may be used for predicting its behavior in real conditions.

Keywords: *Geotrichum candidum*; lactic acid bacteria; growth modelling

Geotrichum candidum is a filamentous yeast-like fungus often present in milk and dairy products such as cream, curd cheese and cheese. For this reason it can be considered as a real milk mould (Kocková-Kratochvílová 1990; Boutrou & Guéguen 2005). This fungus was isolated from milk as early as in 1850 by Fresenius and classified as *Oidium lactis*. Finally it was reclassified to the genus *Geotrichum* Link and received its present name (Wouters *et al.* 2002). *G. candidum* is an anamorphic or a nonsexual microorganism included in the *Hemiascomycetes*. The teleomorphic state of the fungus belongs to the genus *Galactomyces* Redhead and Malloch (Kocková-Kratochvílová 1990; Pottier *et al.* 2008).

G. candidum can grow in a wide spectrum of environmental conditions, particularly temperature and pH. It can grow at the temperature ranging from 5 to 38°C, with an optimum around 25°C. Similarly growth was observed in the large interval of pH values ranging from 3 to 11 with the optimum from 5.0 to 5.5. The generation time of *G. candidum* belongs to the lowest among the eukaryotes (1.1 h at 30°C in liquid medium). It grows as well in the microaerophilic conditions. However, the cultivation in an oxygen poor atmosphere induced an elongation of hyphae and a loss of the lateral branches. *G. candidum* has long been known for its sensitivity to salt but this is a strain dependent property (Boutrou & Guéguen 2005).

With respect to the above mentioned growth potential it is not surprising that this filamentous fungus is a natural part of the microflora of raw milk and it could be commonly isolated from cheese produced from raw cows', ewes' and goats' milk (Tornadijo *et al.* 1998; Erdogan *et al.* 2003; Fadda *et al.* 2004; Boutrou & Guéguen 2005; Hayaloglu & Kirbag 2007; Godič Torkar & Vengušt 2008). On the other hand, the presence of *G. candidum* in pasteurised milk and dairy products results from re-contamination of materials during production (Boutrou & Guéguen 2005; Godič Torkar & Vengušt 2008). A role of *G. candidum* in food could be evaluated posi-

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tive but also negative. It is widely used in a milk industry as a second/adjunct culture in many soft cheeses such as Camembert, semi-fresh and semi-hard cheeses (Addis *et al*. 2001; Marcellino *et al*. 2001; Boutrou & Guéguen 2005; Pottier *et al*. 2008). The metabolic activity of *G. candidum* plays an important role in the development of characteristic flavours, taste and other qualities of milk products (Marcellino *et al*. 2001). On the other hand, *G. candidum* is responsible for the degradation of fresh cheeses, fruit juices and vegetables (Laurenčík *et al*. 2008). Various species of genus *Geotrichum* cause the spoilage of some cream cheeses (Chapman & Sharpe 1990), and can be responsible for spoiling butter, cream and cream products (Varnam & Sutherland 1994).

The ewes' lump cheese is a traditional Slovak milk product widely used for production of bryndza, an artisanal Slovak soft spreadable cheese. The frequent occurrence of *G. candidum* in bryndza was confirmed by Laurenčík *et al*. (2008). In this study it was recorded in every tested sample of cheese.

The aim of this study was to analyse the growth dynamics of *G. candidum* strain isolated from ewes' lump cheese during the co-cultivation with lactic acid bacteria, the Fresco culture, and to compare the growth kinetics of *G. candidum* in milk and on the surface of the skim milk agar (SMA) during both types (single and co-culture) of the cultivation. Basically, the presence of *G. candidum* in fresh cheese (cottage cheese or quark) is undesirable so the study of the growth dynamics of *G. candidum* in the co-cultivation with Fresco can explain a possibility to control the fungus growth.

MATERIALS AND METHODS

Microorganisms. The yeast-like fungus *Geotrichum candidum* was isolated from ewes' lump cheese using the glucose-yeast extract-chloramphenicol agar (YGC, Imuna, Šarišské Michaľany, Slovakia). An identification based on the morphological characteristic was done by Dr. E. Piecková, MPH (Slovak Health University, Bratislava). The strain of *G. candidum* was maintained on slopes of skim milk agar (SMA, Merck, Darmstadt, Germany) at 5 ± 1°C.

The Fresco, commercial culture of Christian Hansen (Hørsholm, Denmark), is generally used for the production of cottage cheese. The culture was kept frozen at –25°C until using in the growth experiment.

Milk inoculation and cultivation. Ultra-pasteurised milk with 1.5% fat content (Rajo, Bratislava, Slovakia) was used for cultivation experiments. The standard suspension of *G. candidum* used for milk inoculation was prepared from 48–72 h old culture grown on SMA agar. An initial cell density was adjusted to $N_0 \leq 10^3$ CFU/ml. Prior to co-cultivation, the milk was kept at intended temperature of incubation and then inoculated with both *G. candidum* and 24 h Fresco culture. The initial densities of lactic acid bacteria (LAB) were in the range of 10^6–10^7 CFU/ml. The experiments were performed in 300 ml of UHT milk without shaking, in the aerobic conditions and at the temperatures ranging from 5°C to 37 ± 0.5°C. At all experimental conditions two or three parallel tests were done.

Number of microorganism in ultra-pasteurised milk. The density of *G. candidum* was calculated according to the Slovak Technical Standard STN ISO 7954 as a number of colony-forming units per millilitre of UHT milk (CFU/ml). The density of Fresco culture was determined according to STN ISO 4833 as number of the mesophilic bacteria grew on the M17 agar (Biokar Diagnostics, Beauvais, France).

Inoculation and experimental performance on the surface of SMA agar. The growth dynamics of *G. candidum* was studied also on the surface of SMA agar. After sterilisation, 1% of 24 h old Fresco culture was added into SMA agar. Sterile Petri dishes with internal diameter of 11 cm were used for the cultivation. *G. candidum* was inoculated on the surface of SMA agar immediately after solidification, but also after 24 h and 48 h of incubation at 30°C. The fungus mycelium grown on slope of SMA agar after 48–72 h was used for inoculation of the agar solid medium with LAB. It was transferred into the centre of Petri dish by touching the agar with a microbiological loop. The colony diameter was measured with vernier calliper (150 × 0.02 mm, Jiangsu S. Ltd.) in two orthogonal directions. The final diameter of colonies taken into growth curve was calculated as the arithmetic mean from both data. At the same time we also measured the pH value of agar using pH meter Knick Portamess equipped with sticking electrode Knick SE 104 (Berlin, Germany). Experiments were carried out in triplicates at the same temperatures as in the milk.

Fitting of growth curves and calculating the growth parameters. The growth parameters of *G. candidum* and lactic acid bacteria in milk were calculated using primary modelling technique (Baranyi *et al*. 1993). The same model was used for fitting the growth curves of *G. candidum* growing on the surface of SMA agar. The dependence of the growth rates of yeast-like fungus on the environmental conditions in milk was analysed using the Ratkowsky model:

$$\sqrt{Gr} = b(T - T_{min}) \quad (1)$$

where:

b – regression coefficient (log CFU/ml$^{0.5}$/h$^{0.5}$/°C)
T_{min} – theoretical minimal growth temperature (°C)
Gr – growth rate of microorganism (log CFU/ml/h)

RESULTS AND DISCUSSION

Growth dynamics of *G. candidum* in co-culture with Fresco in milk

The growth curves of *G. candidum* (Figures 1a and 1b) cultivated in the co-culture with Fresco were compared with the single fungus cultivation carried out in the same experimental conditions. In cheese the growth of lactic acid bacteria is followed by filamentous fungi. Therefore, in order to approach real conditions in cheese the Fresco inoculums were $N_0 \geq 1.10^7$ CFU/ml in each experiment. High inoculation level resulted in growth without previous lag-phase (except at 5°C) with subsequent decrease of milk pH (below 4.7).

At the lowest temperature (5°C), the Fresco started to grow after 5.5 days. The growth rate was 0.006 log CFU/ml/h (Table 1). The pH of milk decreased from 6.7 to 4.6. At the same temperature *G. candidum* showed better adaptation, it started to multiply exponentially (Gr = 0.016 log CFU/ml/h) after almost three days long lag-phase and the stationary phase was reached in the tenth day of the cultivation. The growth rate of *G. candidum* in the co-culture was about 24% lower than in the monoculture.

At 10°C Fresco reached a stationary phase in 3 days without previous lag-phase and the pH of milk decreased to final value of 4.5. In the case of *G. candidum* the lag-phase was shorter (86 h) and its growth rate higher (Gr = 0.023 CFU/ml/h) than at 5°C. Also at this temperature the growth of *G. candidum* was slower (44%) in the co-culture with Fresco in comparison with the monoculture. The growth rate of Fresco was four times higher than at 5°C which is connected to the more suitable environmental conditions. Anyway this did not prevent *G. candidum* from the exponential multiplication.

At the temperature of 15°C the growth rate of Fresco was about 1.5 times higher than at 12°C and more than three times higher in comparison with 5°C. The pH of milk decreased to 4.5 in 2 days period. The duration of lag-phase of *G. candidum* was 48 h and the growth rate was about 37% slower than in the monoculture. Similar trends were observed at temperatures ranging from 18°C to 30°C with an acceleration of the growth rate with increasing temperature (Table 1).

At 35°C the Fresco culture reached maximal counts in 6 h and the growth rate was almost 16 times higher in comparison with 12°C. At this temperature an unusual growth of fungus was observed. *G. candidum* started to die after a short lag-phase and its numbers decreased by more than one log order. After 24 h an exponential multiplication started. Initial decrease in the fungus numbers as well as lower growth rate in comparison to 30°C indicated that ist optimal growth temperature has been exceeded.

High temperature and the presence of lactic acid bacteria significantly inhibited the growth of the filamentous fungus that was however, able to adapt to unfavourable environmental conditions, finally. The difference between growth rates of *G. candidum* in the monoculture and in the co-culture with Fresco within temperature interval from 18 to 35°C ranged from 43% to 52%. So in general, the fungus growth in the milk was nearly twice slower in the co-culture than during the single cultivation. The phase of the exponential growth of Fresco at the highest temperature (37°C) lasted for 6 h and in the meantime the pH decreased to 4.4. Like at 35°C also at this temperature *G. candidum* started to die with the rate of –0.038 log CFU/ml/h. In 3 days the fungus counts decreased to values lower than 10 CFU/ml. According to literature, the temperature of 38°C was found as maximum for *G. candidum* growth (Boutrou & Guéguen 2005). This fact seems to be in agreement with growth dynamics presented hitter-to.

The growth rates of *G. candidum* calculated from D-model (Baranyi *et al*. 1993) were modelled in relation to the cultivation temperature using the Ratkowsky square root model (Figure 2). The re-

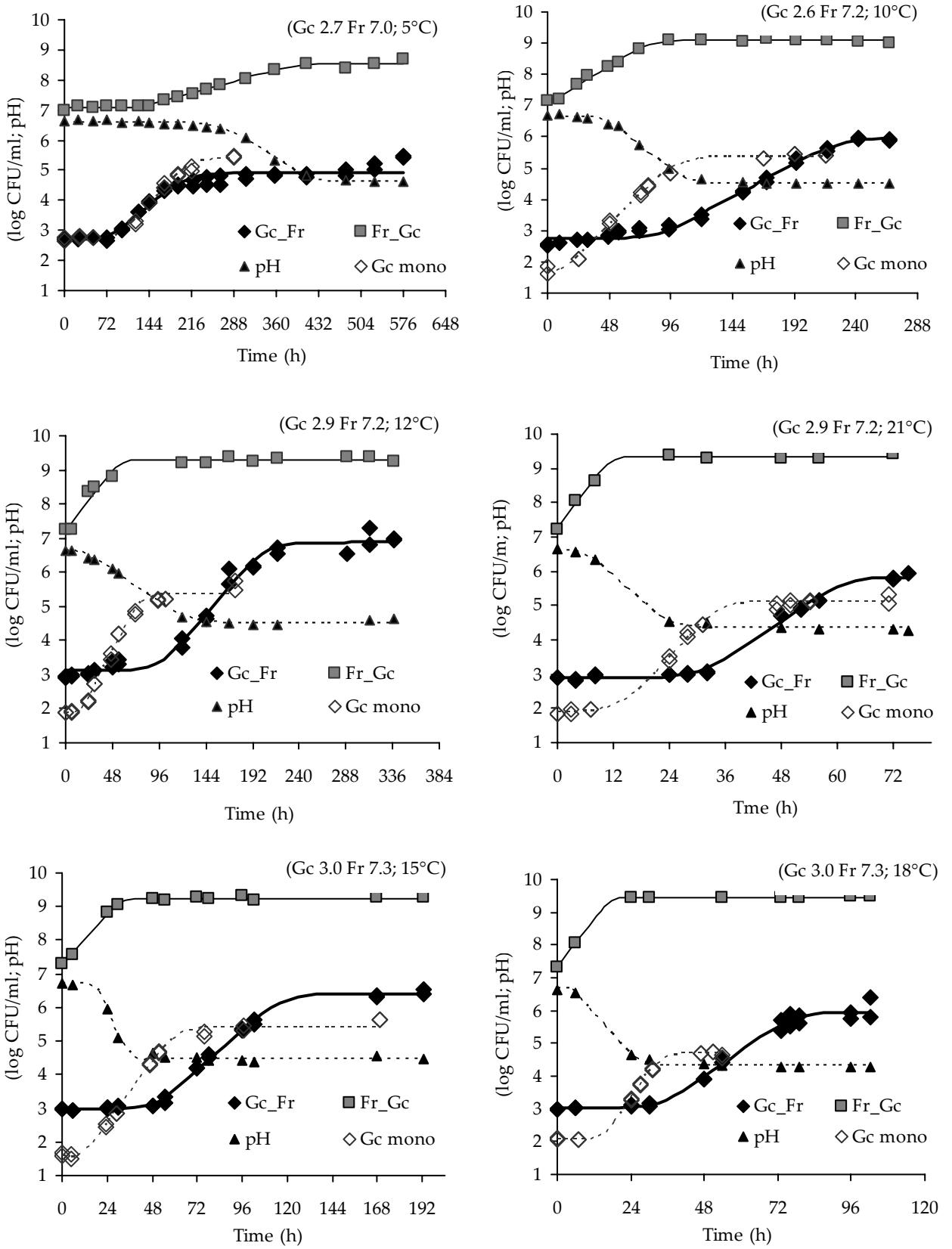

Figure 1a. Growth curves of *G. candidum* and Fresco during their mixed cultivation in milk (Gc_Fr is growth dynamics of *G. candidum* during mixed culture; Fr_Gc is growth dynamics of Fresco during mixed culture; Gc mono is growth dynamics of *G. candidum* during its monoculture in milk)

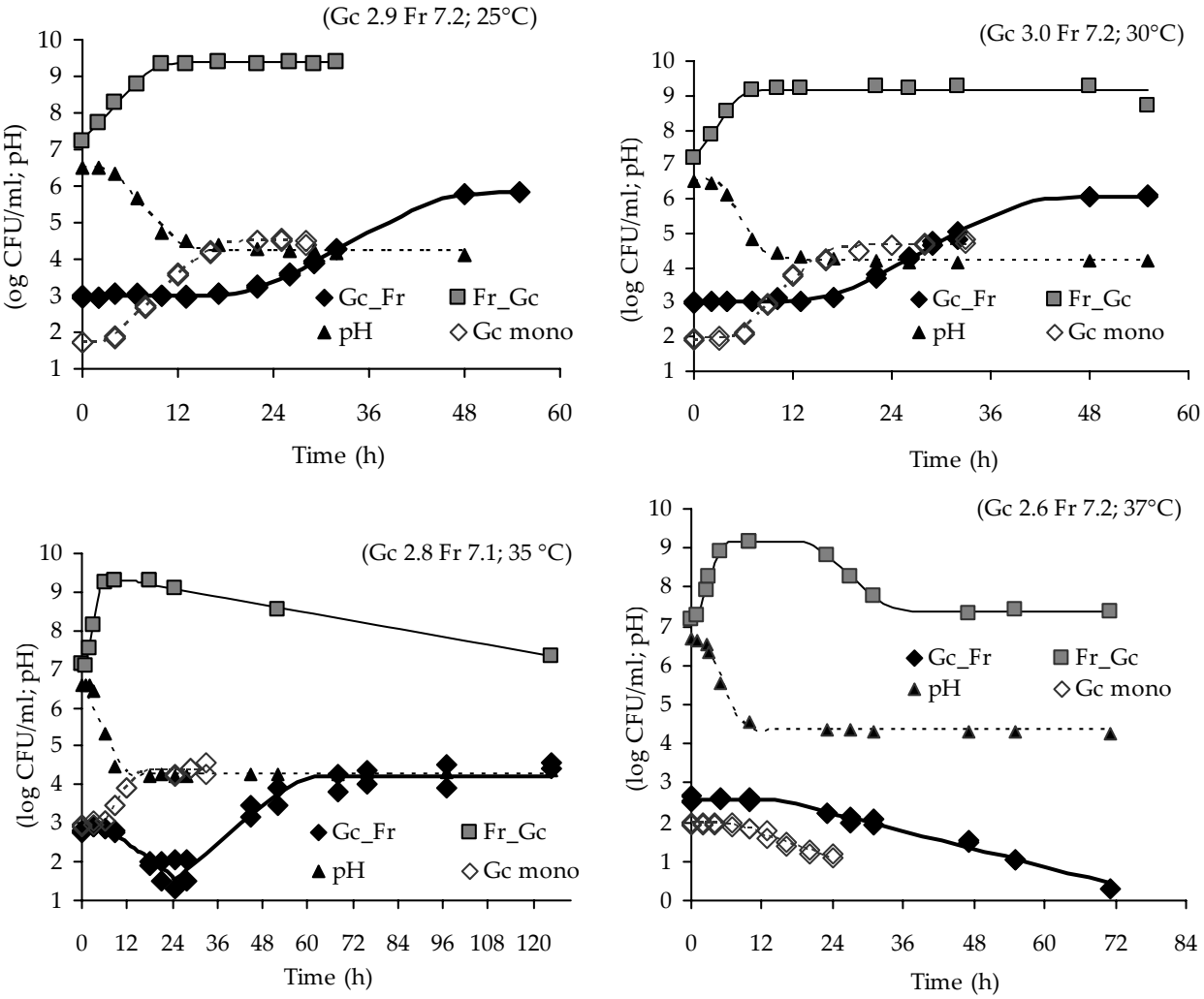

Figure 1b. Growth curves of *G. candidum* and Fresco during their mixed cultivation in milk (Gc_Fr is growth dynamics of *G. candidum* during mixed culture; Fr_Gc is growth dynamics of Fresco during mixed culture; Gc mono is growth dynamics of *G. candidum* during its monoculture in milk)

sults are summarised by the following equations; (2) for mono- and (3) for co-cultivation:

$$\sqrt{Gr} = 0.0148\ (T - T_{min}) + 0.1292$$
$$R^2_{\sqrt{Gr}} = 0.9714 \quad (2)$$

$$\sqrt{Gr} = 0.0106\ (T - T_{min}) + 0.1068$$
$$R^2_{\sqrt{Gr}} = 0.9608 \quad (3)$$

The filamentous fungus had to compete for nutrients and space which have finally resulted in the deceleration of the growth. But anyway, the presence of lactic acid bacteria did not prevent its exponential multiplication. Lactic acid bacteria are able to assimilate nutrients from surrounding medium and produce substances such as weak organic acids (lactic and acetic acids), phenyllactic and pyroglutamic acids, carbon dioxide, hydrogen peroxide, that are often harmful for other microorganisms (Magnusson *et al.* 2003; De Muynck *et al.* 2004; Liptáková *et al.* 2007, 2009; Voulgari *et al.* 2010). Weak organic acids are antimicrobialy active only in the undissociated form, the concentration of which in medium is dependent on the pH. During the experiments the pH of milk decreased to almost 4.5. The bacteria species present in Fresco culture transform lactose exclusively to lactic acid. At low pH of milk, the hydrophobic undissociated form of lactic acid penetrated into the cells of fungus. This was followed by their dissociation and the decrease

Table 1. Growth parameters of *G. candidum* during single and co-culture with Fresco in UHT milk

T (°C)	Gr_{Gc_Fr}	Gr_{Gc_mono}	Gr_{Fr_Gc}	$Gr_{\_pH}$	$N_{max\_Gc\_Fr}$	N_{max_Gc}
	(log CFU/ml/h)				(log CFU/ml)	
5	0.016	0.021	0.006	–0.014	4.90	5.43
10	0.023	0.041	0.026	–0.032	5.98	5.69
12	0.034	0.056	0.039	–0.020	6.87	5.35
15	0.051	0.081	0.064	–0.145	6.40	5.23
18	0.066	0.138	0.123	–0.107	5.96	4.68
20	–	0.154	–	–	–	5.09
21	0.093	–	0.179	–0.117	5.83	–
25	0.118	0.208	0.231	–0.253	5.84	4.49
30	0.129	0.239	0.333	–0.407	6.08	4.64
35	0.083	0.151	0.615	–0.363	4.24	4.36
37	–0.038	–0.051	0.453	–0.381	–	–

Gr_{Gc_Fr} is growth rate of *G. candidum* in the co-culture, Gr_{Gc_mono} is growth rate of *G. candidum* in the monoculture, Gr_{Fr_Gc} is growth rate of Fresco in the co-culture, $Gr_{\_pH}$ is rate of pH decrease in the co-culture, $N_{max\_Gc\_Fr}$ are the final numbers of *G. candidum* in the co-culture and N_{max_Gc} are final numbers of *G. candidum* in the monoculture in milk

of the intracellular pHi. As a result of this action the growth rate of fungus decreased. Yeasts are able to reduce the accumulation of weak acids inside the cells. The mechanisms of weak acid adaptation in yeasts are described in the studies of Brul and Coote (1999), Piper *et al.* (2001), Hazan *et al.* (2004).

Growth dynamics of *G. candidum* during co-culture with Fresco on the surface of SMA agar

Fungal growth dynamics performed on the solid surface of milk agar inoculated with LAB was analysed within the second part of the study. In

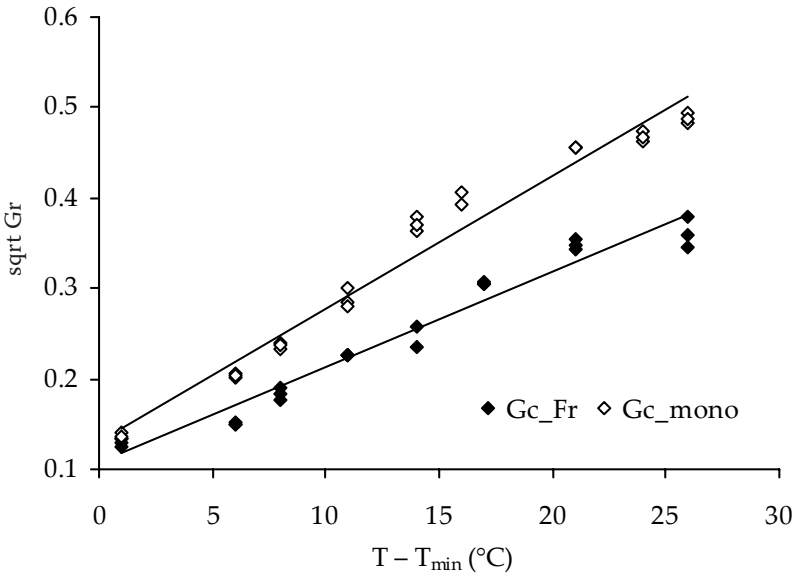

Figure 2. Ratkowsky model designed for growth rate of *G. candidum* during its co-culture (Gc_Fr) and monoculture (Gc_mono) in milk

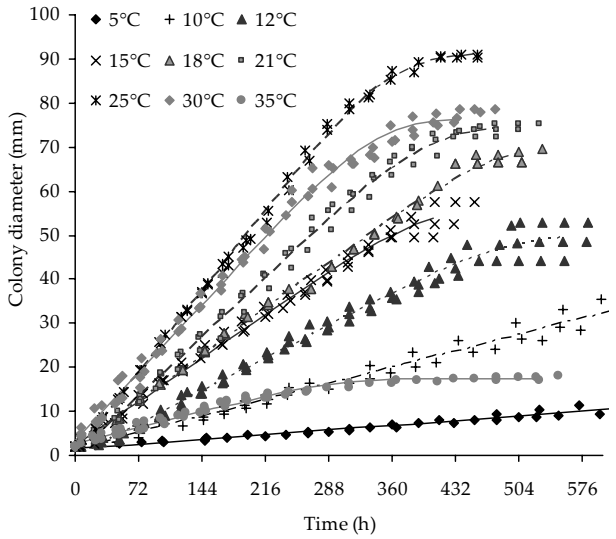

Figure 3. Growth dynamics of *G. candidum* during mixed culture with Fresco on the surface of SMA agar

the majority of experiments, *G. candidum* began to multiply without previous lag-phase, which was probably caused by high amount of exponentially growing cells inoculated on the agar surface. The exponential growth was detected in nearly each trial and the growth rate increased with the temperature until 25°C (Figure 3). No growth of *G. candidum* was observed at 37°C. The lactic acid bacteria were able to reduce pH to the values ranging from 5.4 to 4.0 during the pre-incubation and as well during the actual cultivation. Pre-incubation of the plates with LAB at 30°C did not show any significant effect on the *G. candidum* growth. Therefore, all the growth data from the parallel trials were used for the fitting of fungal growth curve within primary modelling.

At 5°C, the growth rate of *G. candidum* reached the value of 0.015 mm/h. The fungus growth rate increased with temperature and the maximal growth rate was recorded at 25°C (Gr_r = 0.257 mm/h). At this temperature the fungus was able to grow 2.5 times faster in comparison with 12°C and 17 times faster than at 5°C. Further temperature increase to 30°C did not lead to acceleration of the fungus multiplication. The growth rate of *G. candidum* decreased by 17% in comparison to 25°C ($Gr_{30°C}$ = 0.214 mm/h) and this trend continued to the rate of 0.049 mm/h also at 35°C what meant decrease in 81% from the maximum.

At each cultivation temperature, the growth rates of *G. candidum* observed during the co-cultivation and the single cultivation on the surface of SMA agar were very close. The 17% difference was recorded at 5°C, at 10°C it was 25% and at 12°C and 25°C only 16%. At 15°C and at 35°C the difference between the growth rates in mono- and co-cultivation did not exceed 10% (10% at 10°C and 8% at 35°C). These results indicate that the co-cultivation with Fresco did not have any significant effect on the growth rate of filamentous fungus.

On the other hand, the temperature showed high significant linear effect on both radial growth rates of *G. candidum* determined during mono- (Eq. 4) and co-cultivation (Eq. 5) with LAB of the Fresco culture (Figure 4):

$$Gr_{rad\_Gc} = 0.0098T - 0.0338$$
$$R^2_{Gr_{rad}} = 0.9800 \quad (4)$$

$$Gr_{rad_{Gc\_Fr}} = 0.0115T - 0.047$$
$$R^2_{Gr_{rad}} = 0.9687 \quad (5)$$

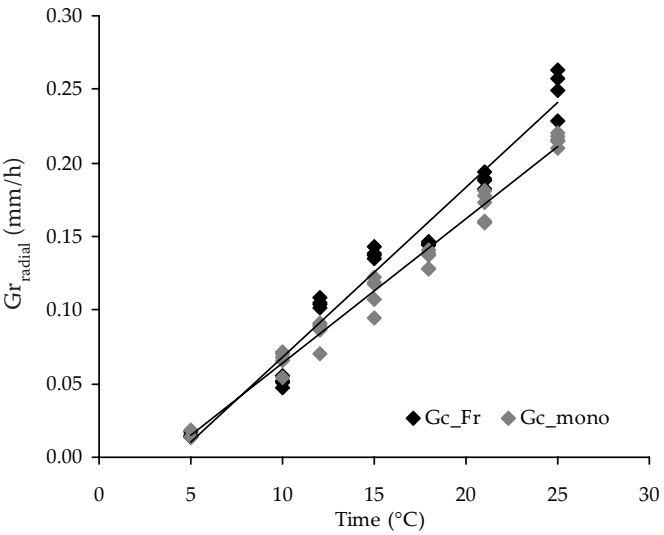

Figure 4. *G. candidum* radial growth rate as a function of temperature during mono- and co-cultivation with Fresco on the surface of SMA agar

The growth of *G. candidum* was not affected by the presence of Fresco culture. Fungus grew during the co-culture as well as during the single culture. These results indicate a good adaptation of fungus to the tested environmental conditions. The strain of *G. candidum* was originally isolated from ewe's lump cheese, where it develops on the surface of the cheese. On the surface of agar its growth was favoured by higher oxygen availability. And what more, *G. candidum* is able to assimilate the lactic acid as a source of energy (Boutrou & Guéguen 2005), which resulted in an increase of pH. Due to growth and assimilation of lactic acid by *G. candidum*, increase of pH of agar from 4.5 to more than 7.0 was observed. Together with the changing of majority form of lactic acid in less effective dissociate one the inhibitive potential of lactic acid bacteria can be limited.

According to obtained results we could conclude that filamentous fungus grew better on the surface than in liquid medium. This can be explained with the fact that oxygen accessibility was decreased during growth of *G. candidum* in milk. This naturally had no effect on growth and the lactic acid production by facultative anaerobic LAB and thus it seemed that their competitive and inhibitory effect acted on the fungal growth additionally. Advantages of solid cultivation of the fungi in comparison with liquid broth are discussed in study of Viniegra-Gonzáles *et al*. (2003).

Validation of secondary models

The secondary model (Eqs 2 and 3) and simple linear function (Eqs 4 and 5) were validated according to Baranyi *et al*. (1999) at all cultivation conditions. The accuracy factor, bias factor and percentage of discrepancy were calculated using following equations:

$$B_f = \exp\left(\frac{\sum_{k=1}^{m}(\ln f(x^{(k)} - \ln \mu^{(k)})}{m}\right) \quad (6)$$

$$\%D_f = (A_f - 1) \times 100\% \quad (7)$$

$$A_f = \exp\left(\sqrt{\frac{\sum_{k=1}^{m}(\ln f(x^{(k)} - \ln \mu^{(k)})^2}{m}}\right) \quad (8)$$

where:

A_f – accuracy index
B_f – bias index
$\%D_f$ – per cent discrepancy
$f(x^{(k)})$ – growth rate calculated from model
$\mu^{(k)}$ – measured growth rate and m is a number of measures

The calculation of accuracy and discrepancy factors were based on the comparison of the growth rates recorded during the experiments and growth rates calculated using the Eqs 2 to 5. The results of the validation are summarised in Table 2.

The B_f value equal to 1 indicates perfect agreement between predictions and observations. The bias factor lower than 1 indicates that the use of the model for the predictions is, in general, fail safe, but Valík *et al*. (2008) considered that values of B_f ranging from 0.9 to 1.05 still represent good model performance. A_f index equal to 1 indicates a perfect agreement between all predicted and observed values. The higher the value the less accurate is the estimate. As can be seen from Table 2, B_f and A_f values calculated for the secondary model and simple linear function are close to 1 at all cultivation conditions. Percentage of discrepancy reached values of 12.8% for the single and of 15.9% for the co-cultivation in milk. $\%D_f$ between the observed

Table 2. The validation parameters defined by Baranyi *et al*. (1999) for secondary model and for simple linear function represented by mentioned equations

	Validation index for equation			
	2	3	4	5
A_f	1.128	1.159	1.086	1.214
B_f	1.017	1.008	0.999	0.970
D_f	12.8	15.9	8.6	21.4
R^2	0.9714	0.9608	0.9800	0.9687

and the predicted growth rates recorded on the surface of SMA agar reached the values of 8.6% in the mono- and of 21.4% in the co-culture. In our opinion, in the case of the microbial growth these values of the validation parameters are still acceptable.

Similar values of the validation parameters were found by Liptáková *et al.* (2007), who used the Ratkowsky model to describe *Candida maltosa* growth in UHT milk. The authors reported the accuracy factor ranging from 1.04 to 1.23 and bias factor from 1.00 to 1.04 for the yeast growth rate during the co-cultivation with the probiotic culture of *Lactobacillus rhamnosus* VT1. Valík and Piecková (2001) modelled the radial growth of the heat-resistant filamentous fungi cultivated on the surface of agar with A_f and B_f values ranging from 1.070 to 1.106 for A_f and from 1.007 to 1.019 for B_f. Samapundo *et al.* (2005) recorded the values of discrepancy between the observed and the calculated growth rates lower than 13% for *Fusarium verticilliodes* and lower than 16% for *F. proliferatum*.

CONCLUSION

According to our results the growth dynamics of the *G. candidum* was partially inhibited during the co-cultivation with LAB in milk. On the other hand, in real aerobic system which was represented by surface of milk agar the growth of *G. candidum* has similar rate during both, co-culture and single cultivation in the whole temperature range used within the experiments. The precise data describing the growth of this cheese yeast-like fungus, *G. candidum,* may fill a gap in the field of quantitative food mycology and may be used for predicting its behavior in real conditions. Our results have confirmed that the growth of *G. candidum* can be controlled using lactic acid bacteria during a fermentation of milk. On the contrary, the control of its growth e.g. with the help of LAB was not effective on the surface of milk agar medium. In order not to convert a positive role of *G. candidum* to a role of the food contaminant in ripening of some sorts of cheese it is extremely important to use a surface salting. The surface salting is also used during the artisanal production of fresh or shortly ripened ewes' lump cheese in traditional Slovak 'salashes'.

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