Effect of Temperature and Lactic Acid Bacteria on the Surface Growth of *Geotrichum candidum*

Anna HUDECOVÁ[1], Ľubomír VALÍK[1], Denisa LIPTÁKOVÁ[1], Jana PELIKÁNOVÁ[1] and Michal ČIŽNIAR[2]

[1]Department of Nutrition and Food Assessment and [2]Department of Information Engineering and Process Control, Institute of Biochemistry, Nutrition and Health Protection, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic

Abstract

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The surface growth of *Geotrichum candidum* isolated from ewes' lump cheese was studied on pure agar medium and that inoculated with *Lactobacillus rhamnosus* GG and *L. paracasei* subsp. *paracasei* CCM 1753. The colony growth rates of fungus calculated from the growth curves were modelled in relation to temperature by the cardinal temperature model with inflection (CTMI). The following cardinal values resulted from the secondary model: T_{min} = –3°C, T_{opt} = 27.6°C, and T_{max} = 35.4°C and optimal colony growth rate μ_{opt} = 5.34 mm/day. A quantitative study also showed that the simultaneous growth of *L. rhamnosus* GG and *L. paracasei* subsp. *paracasei* CCM 1753 had either no or only a slight effect on the fungal growth rates, respectively. These results pointed out that other intrinsic or extrinsic factors should be applied for the protection of fresh cheeses against the undesirable growth of *G. candidum.*

Keywords: *Geotrichum candidum*; surface growth; CTMI model; lactic acid bacteria

Geotrichum candidum is a ubiquitous fungus found in various habitats such as soil, air, water, plants, animals, and humans. This fungus occurs commonly in raw milk and milk products, and its designation as a real milk mould is also used (Jodral *et al*. 1993; Wouters *et al*. 2002; Görner & Valík 2004). The role of *G. candidum* in the dairy environment is disputable. It is considered a spoilage agent of a number of dairy products like cheese, fermented milk, butter and cream (Varnam & Sutherland 1994; Botha 2000; Ledenbach & Marshall 2009). On the other hand, it is used as a secondary culture in the production of specific types of cheese varieties (Pottier *et al*. 2008).

G. candidum reproduces through the fragmentation of vegetative hyphae and production of arthrospores (Caldwell & Trinici 1973; Kocková-Kratochvílová 1990). This fungus is relatively resistant against some unfavourable conditions like low temperature, pH, and low concentration of oxygen. *G. candidum* grows under microaerophilic conditions. The cheese interior is essentially an anaerobic system and the fungus is able to grow not only on the surface of but also inside the cheese, although at hundred times lower concentration (Haasum & Nielsen 1998; Boutrou & Guéguen 2005).

G. candidum is a common part of raw milk cheese microflora. In spite of this, there is a lack of precise

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quantitative data concerning the fungus growth in such an environment. When the fungus grows on the cheese surface, the white coat formation can be observed. In cheese, the overgrowth of fungus is responsible not only for changing the appearance but also for the catabolism of milk protein and fat (Marcellino *et al*. 2001). In the dairy practice, it is important to keep the growth of *G. candidum* under the desired level and to control in this way the flavour development.

The aim of this work was to study the growth dynamics of *G. candidum* on the surface of milk agar in relation to temperature. The impact of the competitive microflora represented by *Lactobacillus rhamnosus* GG and *L. paracasei* subsp. *paracasei* CCM 1753, respectively, on the surface growth of fungus was also studied.

MATERIAL 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, Slovak Rwpublic). The phenotypic identification of the fungus was confirmed by Assoc. Prof. E. Piecková, MPH, PhD. (Slovak Health University, Bratislava). The fungus isolate was maintained on the slope of skim milk agar (SMA, Merck, Darmstadt, Germany) at 5 ± 1°C. The probiotic strain *Lactobacillus rhamnosus* GG was obtained from Dr. Salminen (University of Turku, Turku, Finland) through the mediation of Dr. Lauková (State Veterinary and Food Institute, Košice, Slovak Republic). *Lactobacillus paracasei* subsp. *paracasei* 1753 was purchased from the Czech Collection of Microorganisms – CCM (Brno, Czech Republic). Lactobacilli were stored in Man-Rogosa-Sharpe broth (MRS, Biokar Diagnostics, Allonne, France) at 5 ± 1°C.

Agar preparation, inoculation and culture conditions. The growth dynamics of *G. candidum* was studied on the surface of SMA agar. Sterile agar was poured into Petri dishes with an internal diameter of 11 cm. After gelling, the fungus was inoculated into the centre of the agar plate. For inoculation purposes, 48–72 h old culture of *G. candidum* grown on SMA agar slope was used. The fungus culture was transferred into the centre of each Petri dish by touching the agar with a microbiological loop. The concentration of the fungus inoculum was $6.0 \times 10^5 \pm 2.0 \times 10^5$ CFU per microbiological loop (ν_k = 33%). After inoculation, the subsequent aerobic cultivation of the agar plates was carried out at temperatures ranging from 5 to 37°C. The growth of the fungus was studied at various pH values ranging from 5.0 to 7.0 with steps of 0.5 at temperatures from 10 to 25°C, and at pH of 5.5 and 7.0 at temperatures of 5, 30, 35, and 37°C. The pH of the agar medium was adjusted with lactic acid. The diameter of the fungus colony was measured with vernier calliper (15 × 0.02 mm; Jiangsu S. Ltd., China) in two orthogonal directions. The final colony diameter was calculated as the arithmetic mean. The pH value of agar was measured at the beginning of and occasionally during the experiment using pH meter Knick Portamess equipped with the sticking electrode Knick SE 104 (Berlin, Germany). Each set of experiments was done in triplicate.

In the co-cultures with lactobacilli 1% (v/v) inoculum of the bacteria was added into cooled SMA agar before pouring it into Petri dishes. As the inoculum, 24 h old culture of lactobacilli grown in 10 ml MRS broth was used. Before the fungus inoculation, the incubation of the agar plates with lactobacilli was performed at 37°C for 48–72 hours. After the fungus surface inoculation, the incubation of the agar plates proceeded in the following way: The fungal colony diameter measurements were performed during the growth on agar containing *L. rhamnosus* GG at the same temperature intervals as in the cultivation of *G. candidum* without bacteria. The influence of *L. paracasei* subsp. *paracasei* 1753 on the fungus surface growth was investigated at 5, 8, 10, and 12°C.

Microbial modelling. The diameter of *G. candidum* colonies as a function of time was modelled using DMFit model proposed by Baranyi *et al*. (1993). The maximum growth rate acquired from the growth curve and expressed as the increment of the colony diameter over time (μ_{max}) was subjected to the secondary modelling in relation to the incubation temperature. For this purpose we used the cardinal model with inflection (CTMI) firstly introduced by Rosso *et al*. (1993):

$$\mu_{max} = \frac{\mu_{opt}(T - T_{max})(T - T_{min})_2}{(T_{opt} - T_{min})[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)]} \quad (1)$$

where:

T_{min} – temperature below which no growth occurs (°C)

T_{max} – temperature above which no growth could be observed (°C)

T_{opt} – temperature (°C) at which the maximum growth rate reached its optimal value μ_{opt} (mm/day)

Validation of the growth predictions. The goodness of fit of the secondary model (Eq. 1) was assessed by the root mean square error (RMSE):

$$RMSE = \sqrt{\frac{\sum \mu_{observed} - \mu_{predicted}}{n - p}} \quad (2)$$

where:

$\mu_{observed}$ – maximum growth rate obtained from the primary curve fitting

$\mu_{predicted}$ – maximum growth rate computed from the applied secondary model

n – number of observations

p – number of parameters to be estimated

The validation of the model was performed in compliance with Ross (1996). The validation indices as the accuracy factor and bias factor were calculated:

$$A_f = 10^{\left(\frac{\sum|\log(\mu_{predicted}/\mu_{observed})|}{n}\right)} \quad (3)$$

$$B_f = 10^{\left(\frac{\sum\log(\mu_{predicted}/\mu_{observed})}{n}\right)} \quad (4)$$

where: $\mu_{observed}$, $\mu_{predicted}$, and n see Eq. 2

RESULTS AND DISCUSSION

Growth dynamics of *G. candidum* on the surface of SMA agar

The growth of *G. candidum* was studied at temperatures ranging from 5 to 37°C. This interval was selected in order to cover the entire growth area of this microorganism. The growth of *G. candidum* on the surface of the agar medium followed a sigmoidal curve, but in a majority of experiments the lag-phase was missing. The absence of the lag-phase was most likely caused by the high concentration of fungus inoculum on the surface of the same medium. Maximum growth rate and maximum colony diameter of *G. candidum* were estimated through the primary model (Table 1).

Within the tested range of temperature, an insignificant effect of medium pH on the surface growth of *G. candidum* was observed. The same result was obtained in our previous work concerning the fungus growth at suboptimal temperatures and within pH range of 5.0–7.0 (Hudecová *et al.* 2008). The growth of *G. candidum* could be observed in a wide pH range of 3 to 11 while its optimal pH is referred to be 5.5–6.0 but also 6.0–7.0. With respect to the above data and in agreement with Tempel and Nielsen (2000) and Boutrou and Guéguen (2005), it could be concluded that pH commonly applied in the manufacture of cheese and ranging from 4.4 to 6.7 would have a minor effect on the growth of the fungus strains isolated from this kind of food product.

Table 1. Maximum growth rate (μ_{max}) and final colony diameter (d_{end}) of *G. candidum* on SMA agar at pH of 5.5 and 7.0 in relation to temperature

T (°C)	$\mu_{max\ 5.5}$ (mm/day)	$d_{end\ 5.5}$ (mm)	$\mu_{max\ 7.0}$ (mm/day)	$d_{end\ 7.0}$ (mm)
5	0.43	–	0.34	–
8	0.97	46.8	0.91	41.6
10	1.59	64.2	1.31	55.8
12	2.14	68.6	2.17	67.8
15	2.85	72.4	2.28	70.1
18	3.07	103.6	3.08	114.4
20	3.85	69.5	3.82	72.5
25	5.05	65.8	5.19	61.9
30	5.13	50.4	5.71	44.4
35	1.16	20.5	1.06	20.0
37	0.01	–	0.06	5.1

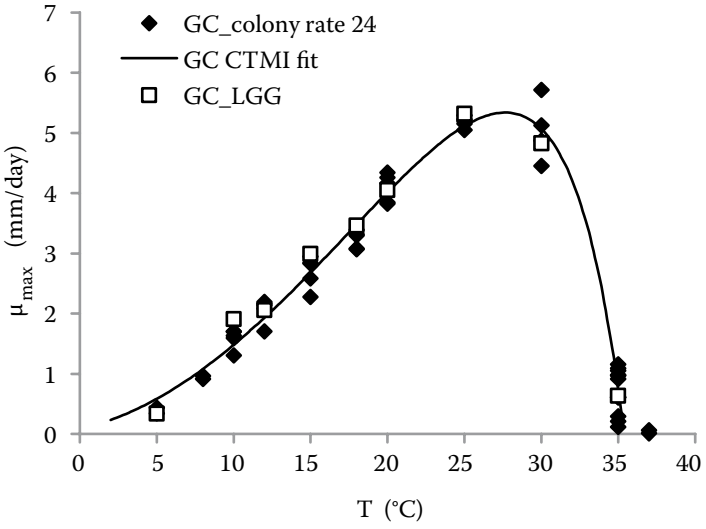

Figure 1. Growth rate of *G. candidum* as a function of temperature

GC_colony rate 24 – growth rates of fungus estimated through Baranyi's primary model (Baranyi *et al.* 1993), continuous line indicates the fitted CTMI model (Eq. 1); GC_LGG – growth rates of fungus in the co-culture with *L. rhamnosus* GG used for external validation

On the contrary, the incubation temperature influenced the growth significantly. The effect of temperature on the fungus growth rate regardless of pH was described with CTMI model (Figure 1). The cardinal temperatures and the optimal growth rate for *G. candidum* are shown in Table 2. The minimum temperature was observed to be close to the freezing point. This value is below 5°C which is reported as minimum in the literature (Boutrou & Guéguen 2005). However, the studied isolate of *G. candidum* was still able to grow at 5°C in our research. CTMI model was used also for predicting the fungus growth in milk (Hudecová *et al.* 2010). In this study the calculated minimum reached the values of 1.2°C and 1.4°C depending on the respective pH. The observed optimum temperature falls into the interval reported in literature (Samson *et al.* 1981; Boutrou & Guéguen 2005), however, it was lower than that in the milk. According to Pottier *et al.* (2008) *G. candidum* is not able to grow at 40°C and the maximum temperature is referred to be 38°C. In spite of the weak growth of the fungus on the surface of SMA agar at 37°C, maximum temperature derived from the model was 35.36°C. In UHT milk, the calculated maximum was 35.3 and 37.3°C depending on pH. This led us to the conclusion that also the character of the growth medium affects the resulting growth response of *G. candidum*.

Table 2. Estimated values of the cardinal temperatures and optimal growth rate of *G. candidum* on SMA agar

Parameter	Estimated value ± SE	RMSE	R^2
μ_{opt}	5.34 ± 0.09	0.2848	0.972
T_{min}	–3.02 ± 0.03		
T_{opt}	27.65 ± 0.07		
T_{max}	35.36 ± 0.01		

SE – standard error derived from non-linear regression; R^2 – correlation coefficient

Effect of LAB on the surface growth of *G. candidum*

As the growth of the fungus commonly occurs on the surface of cheese made from raw milk, in the next set of experiments the surface growth of *G. candidum* was studied on SMA agar with the inoculated culture of lactobacilli (Table 3). For this purpose, two species were selected. The first one was *L. rhamnosus* GG, which is a widely studied bacterium because of its claimed probiotic effect on the human health (Servin 2004; Saxelin *et al.* 2005; Sánchez *et al.* 2009). The second one was *L. paracasei* subsp. *paracasei*, which is a common part of the NSLAB microflora of cheese, strains of this species have been proposing for use as probiotics (Jahreis *et al.* 2002; Marzotto *et al.* 2006; Tsai *et al.* 2008). As the fungal growth usually occurs on the surface after the bacterial growth, a high inoculum of lactobacilli was used. It was about 10^6 CFU/ml for *L. rhamnosus* and 10^7 CFU/ml for *L. paracasei*. To favour the bacteria, 48–72 h long cultivation of agar with lactobacilli at 37°C was performed before the fungus inoculation.

The effect of temperature on the surface growth of *G. candidum* in the co-culture with *L. rhamnosus* GG

was similar as that during the single cultivation. According to the DMFit model, the calculated maximal growth rate of the fungus increased with temperature up to 25°C. At this temperature, the maximal growth rate achieved its highest value. With further increase in temperature, the fungus growth decreased sharply and at 37°C no more growth was detected (Table 3). In order to utilise in practise the secondary growth model developed in this study, the fungus growth rate predictions (Table 2) were compared with those measured in the presence of LAB. In this way, the effect of bacteria on the fungal growth was assessed. At temperatures ranging from 12 to 35°C, the observed difference between the growth rates of the fungus in the single culture and in the co-culture with *L. rhamnosus* GG varied only from 1 to 13%. In most cases, the growth of the fungus in the presence of the probiotic strain was even faster. At 5°C, the fungus growth in the single culture was higher and differed from that in the co-culture by 42%, although at this temperature the model predicted a higher growth than that actually observed. When that observed one was used for the comparison, the resulting difference was smaller by half. At 10°C, the opposite situation occurred and the growth of the fungus in the co-culture was higher than that obtained during the single growth with the resulting difference of 22%. At this temperature, the model predicted a slightly slower growth in comparison to that observed. According to the above results, it could be concluded that *L. rhamnosus* GG has no significant effect on the surface growth of the fungus at all.

In the co-culture with *L. paracasei*, only a low temperature interval from 5°C to 12°C was applied. The lactobacillus strain was used with the intention to prevent the fungus growth as there are a number of studies in the literature concerning its antibacterial (Topisirovic *et al*. 2006; Bendali *et al*. 2008) and antifungal potential (Atanassova *et al*. 2003; Durlu-Özkaya *et al*. 2005; Voulgari *et al*. 2010). The comparison of the individual fungus growth with that in the co-culture revealed a unified trend of a slight growth rate deceleration during the cultivation with the lactobacillus strain. At the investigated temperature interval the difference between the single culture growth and that in the co-culture ranged from 31% to 9%. The growth rate overestimation at 5°C as caused by the model produced a result different from that obtained if the observed growth rate was used for comparison. In such case, the growth of fungus decreased only by 7%. However, in general growth modelling was not incorrect as the comparisons at the higher temperatures revealed differences from 9% to 24% which were comparable to the observed ones (18–27%).

The different effects of the lactobacilli used in this work on the fungus growth are in accordance with the results of Álvarez-Martín *et al*. (2008) who recorded a different nature of interaction between the tested strains of *G. candidum* and the selected LAB. The interactions were positive or negative

Table 3. Maximum growth rate (μ_{max}) and final colony diameter (d_{end}) of *G. candidum* on the surface of SMA agar inoculated with *L. rhamnosus* GG (Gc_LGG) and *L. paracasei* subsp. *paracasei* CCM 1753 (Gc_LP), respectively

T (°C)	μ_{max,Gc_LGG} (mm/day)	d_{end,Gc_LGG} (mm)	pH_{in,Gc_LGG}	μ_{max,Gc_LP} (mm/day)	d_{end,Gc_LP} (mm)	pH_{in,Gc_LP}
5	0.34	–	4.2	0.40	28.4	5.0
8	–	–	–	0.82	38.4	5.0
10	1.91	57.9	4.2	1.16	51.8	4.2
12	2.05	64.8	4.6	1.76	50.1	4.9
15	2.99	62.2	4.6	–	–	–
18	3.46	82.6	4.8	–	–	–
20	4.05	65.5	4.3	–	–	–
25	5.32	58.8	5.0	–	–	–
30	4.83	49.6	4.3	–	–	–
35	0.63	14.6	4.2	–	–	–
37	–	–	4.3	–	–	–

pH_{in} – pH of SMA agar in the time of *G. candidum* inoculation

depending on the combination used. Such a result is not surprising if the different growth requirements or modes of the studied microorganisms are considered. The weak ability of *L. paracasei* to reduce the growth of *G. candidum* probably resulted from its NSLAB character which may be responsible for its better adaptation to the experimental conditions in contrast to *L. rhamnosus* GG which originated from the human digestive tract. The opposite result was achieved in the milk environment where the fungus growth was inhibited at low temperature by *L. rhamnosus* GG (Hudecová *et al*. 2010) but in the co-culture with *L. paracasei* it remained unaffected (unpublished data). The studied lactobacilli were inoculated into the milk at the same time as *G. candidum*, which was not the case in this study. In the milk study, the results corresponded to the minimum temperature detected for the studied LAB. *L. rhamnosus* GG is able to grow in milk already from 6°C (Valík *et al*. 2008) in comparison with the higher minimum of 11.2°C reported for *L. paracasei* subsp. *paracasei* 1753 (Pelikánová *et al*. 2011). In the milk environment, the growth inhibition of another fungal microorganism *Candida maltosa* in the co-culture with *L. rhamnosus* GG was also observed (Liptáková *et al*. 2010).

Model validation

Fitting the experimental data with the CTMI model was primary evaluated through the root mean square error and the coefficient of correlation (R^2; Table 2). The both values as well as the errors associated with the cardinal model parameters confirmed statistically well performed fitting. As we did not find any comparable data in the available literature, internal validation which involved the bias and accuracy factors was carried out with the resultant B_f of 1.066 and A_f of 1.228. Since these values indicate only how well the model fits the data originally used for its development, the true predictive ability of the model could be assessed only through external validation.

External validation is based on independent data which are not used for the model derivation. Therefore, for the external validation purposes, the data of the fungus growth rate in the co-culture with *L. rhamnosus* GG were used (Figure 1). The data were selected because they span the whole range of temperature for the fungus growth, and because the lactobacillus strain did not affect the fungal growth. The resulting B_f of 1.031 indicates still a good model performance as it fell into the range of 0.9–1.05 reported in the literature (Mellefont *et al*. 2003; Valík *et al*. 2008). Since the error in the growth rate estimates under controlled laboratory conditions is found to be around 10%, the best A_f of 1.1 could be expected (Mellefont *et al*. 2003). With our model, A_f of 1.146 was determined which is in agreement with the previous assumption. The validation indices presented are comparable with other studies concerning the secondary modelling of the surface growth of fungi. Combined effects of temperature and water activity on the growth of fungi were modelled within gamma concept based on the cardinal models by Judet-Correia *et al*. (2010) with the resulting A_f of 1.11–1.29 and B_f of 1.01–1.06. The same modelling approach was applied by Garcia *et al*. (2011) near the growth boundaries for the studied aspergilli with resulting poor goodness of prediction characterised by B_f of 0.12–1.09 and A_f of 1.07–1.72.

CONCLUSION

The aim of this study was to evaluate the growth dynamics of *G. candidum* on the surface of pure agar medium or that inoculated with *Lactobacillus rhamnosus* GG and *L. paracasei* subsp. *paracasei* CCM 1753. The surface growth of the fungus without lactobacilli was modelled as a function of temperature with CTMI model. The predicted growth rate of the fungus was then compared with the observed growth of *G. candidum* in the co-culture with bacteria. As the model overestimates the growth of the fungus at the lowest temperature, this result was not taken into account during the comparison. The growth rate of *G. candidum* was not affected by *L. rhamnosus* GG at all while the co-culture with *L. paracasei* subsp. *paracasei* 1753 led only to the weak growth inhibition. Besides lower predictive ability of the model at the growth boundaries of the studied microorganism, the process of external validation proved it to be a good predictor. These results demonstrated the high adaptability of the fungus to the surface growth even in the presence of competitive microflora, which highlighted the need of keeping the fresh cheese from the initial fungal contamination.

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Corresponding author:

Ing. Anna Hudecová, Slovenská technická univerzita v Bratislave, Fakulta chemickej a potravinárskej technológie, Ústav biochémie, výživy a ochrany zdravia, Radlinského 9, 812 37 Bratislava, Slovenská republika
tel.: + 421 259 325 515, e-mail: xhudecova@stuba.sk