Effect of lactic acid bacteria on the growth dynamics of *Geotrichum candidum* in fresh cheeses during storage

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Summary
The growth dynamics of *Geotrichum candidum*, inoculated into cottage cheese and into fresh soft cheese prepared on a laboratory scale, was examined at low temperatures (5–12 °C). The exponential growth of *G. candidum* was observed in each experiment. The fungus grew rapidly in both fresh cheeses either as a single culture or in co-culture with selected lactic acid bacteria (LAB). Based on the growth rates of single cultures and co-cultures, we found that LAB had no significant effect on the growth of *G. candidum.* The results of this study showed that the yeast-like fungus is well adapted to low temperature and pH commonly encountered in the fresh cheese environment, even when the protective culture was applied to the product.

Keywords
Geotrichum candidum; cheese; spoilage; lactic acid bacteria

Fresh acid-curd cheeses like cottage cheese are produced by coagulation of milk via acidification resulting from the metabolism of lactose by added starter microorganisms. The steps involved in the production of these cheeses are milk pretreatment, slow acidification and gelation (close to the isoelectric point of casein, i.e. pH 4.6), whey separation and curd treatment. In order to obtain the firmer coagula and to minimize casein loss, a small amount of rennet can also be added. This addition is not essential. Fresh cheeses are ready for consumption once the manufacture is complete and generally have relatively low levels of dry matter, fat and protein, and high moisture levels. Cottage cheese as a fresh dairy product is prone to spoilage by yeasts and moulds. Intrinsic factors of the cheese are not harsh enough to inhibit the growth of these detrimental microorganisms and it may occur even at relatively low storage temperature and pH. Growth and metabolism of yeasts and moulds result in undesirable flavours, surface slime and discolouration [1, 2].

Geotrichum candidum is a yeast-like fungus that belongs to *Hemiascomycetes*, which reproduce mainly by arthric conidiogenesis. The latest taxonomic revision of *Geotrichum* and its teleomorphs has shown that *G. candidum* is an anamorph of *Galactomyces candidus* DE HOOG and SMITH [3]. *G. candidum* is an important microorganism in the food industry. It is used in the dairy industry as a secondary culture in the manufacture of certain cheese varieties. On the other hand, it acts as a spoilage agent in a range of food products [4, 5].

G. candidum belongs to the group of microorganisms called "machine moulds" because of its ubiquitous nature. It is associated mostly with air and insufficiently cleaned surfaces that come to contact with the food product. Moreover, the growth of *G. candidum* is neither influenced by low pH nor by microaerobic conditions [6–8].

Nowadays, the preservation of food by lactic acid bacteria (LAB) appears to be a promising alternative to chemical preservatives. LAB produce a number of compounds with antifungal activity such as lactic, acetic, propionic and phenyllactic acids, hydrogen peroxide, diacetyl, peptides or fatty acids. LAB with antimicrobial activity are selected and used as protective cultures that contri-

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bute to the improvement of food safety and quality [9, 10].

With regard to the above, the objective of this work was to describe quantitatively the growth dynamics of *G. candidum* in fresh cheeses e.g. cottage cheese. Co-cultivations of the fungus and selected strains of LAB were also performed in order to find out if there was a possible inhibitory effect on fungal growth.

MATERIALS AND METHODS

Microorganisms and media

The strain of *Geotrichum candidum* used in the study was isolated from ewes' lump cheese, a traditional Slovak cheese made from raw ewes' milk. The phenotypic identification of the isolate was done according to Samson et al. [11], Botha [4] and Kurtzman et al. [12]. Sequencing of D1/D2 domain of 26S rDNA revealed highest affinity of the isolate to the two *G. candidum* strains (JF262190.1, JF262180.1) [13] but the same concordance was observed with *Geotrichum bryndzae* sp. nov. [14]. However, in a recent study of Groenewald et al. [15], *G. bryndzae* was considered as a synonym of *Galactomyces candidus*. Therefore, the strain used in this study was identified as *G. candidum*. The fungal isolate was kept on skim milk agar slants (SMA; Merck, Darmstadt, Germany) at (5 ± 1) °C. A starter culture of mesophilic LAB Fresco DVS 1010 (Christian Hansen, Hørsholm, Denmark) was used to make the fresh cheese under laboratory conditions. The following LAB were used in co-cultures with *G. candidum*: The probiotic strain *Lactobacillus rhamnosus* GG obtained from Dr. Salminen (University of Turku, Turku, Finland) through the mediation of Dr. Lauková (State Veterinary and Food Institute, Košice, Slovakia), *Lactobacillus paracasei* subsp. *paracasei* CCM 1753 purchased from Czech Collection of Microorganisms (CCM, Brno, Czech Republic) and a protective culture HOLDBAC YMB (Danisco, Niebüll, Germany). The protective culture consisted of *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii* subsp. *shermanii*. Lactobacilli were stored in Man-Rogosa-Sharpe broth (MRS, Biokar Diagnostics, Allonne, France) at (5 ± 1) °C and the protective culture was kept frozen at –25 °C.

Culture media

A commercial cottage cheese with a fat content of 42 $g \cdot l^{-1}$ (Rajo/Meggle, Bratislava, Slovakia) and a cows' fresh cheese prepared on a laboratory scale were used as culture media. A process for making the cottage cheese included the addition of protective culture HOLDBAC YMB. The fresh cheese was prepared as follows. UHT milk was inoculated with the starter culture (1%, v/v) and left to stay overnight at 30 °C to sour. In the next step, the formed coagulum was heated to 55 °C in order to promote a better separation of whey. This heating period lasted 1.5–2 h. Finally, the curd was transferred into a gauze cloth and left about 3 h for whey drainage. The finished cheese was divided into 3 portions of 200 g each and placed in sterile beakers covered with aluminium foil. Details of the preparation procedure of the starter culture inoculum are described in the work of Le Marc et al. [16].

Inoculation of the cheese media

Cottage cheese was inoculated with cell suspension of *G. candidum* prepared from a 2- to 3-d-old culture grown on SMA with subsequent rinsing with sterile water and appropriate dilution. The same cell suspension was used to inoculate the fresh cheese immediately after production. The fresh cheese produced under laboratory conditions was used also for binary co-cultures of *G. candidum* and either *Lb. rhamnosus* GG, *Lb. paracasei* subsp. *paracasei* CCM 1753 or the protective culture. Inocula of *Lb. rhamnosus* GG and *Lb. paracasei* subsp. *paracasei* were prepared in the same way. Volumes of 10 ml of UHT milk were inoculated (1%, v/v) with a 24-h-old bacterial culture grown in MRS broth. After incubation for 24 h at 37 °C, the milk culture was used again as an inoculum for the next 10 ml of milk (1%, v/v). The resulting culture was used to inoculate the fresh cheese medium. A few grains of the frozen protective culture were transferred to 500 ml of UHT milk and incubated for 24 h at 33 °C. LAB were inoculated into the fresh cheese at the same time as *G. candidum*. In both cheese media, the initial cell densities of *G. candidum* and LAB were adjusted to $\leq 10^3$ $CFU \cdot g^{-1}$ and $> 10^6$ $CFU \cdot g^{-1}$, respectively.

In the next set of experiments (the so-called pre-incubation experiments), the binary co-cultures of *G. candidum* and LAB were performed in the following way. LAB and the starter culture were inoculated simultaneously into UHT milk for cheese making. The milk was inoculated with 10% (v/v) of *Lb. rhamnosus* GG and *Lb. paracasei* subsp. *paracasei* inocula and only with 1% (v/v) of the protective culture inoculum. All types of LAB inocula were prepared in the same way as described above. After the fresh cheese production, either *Lb. rhamnosus* GG, *Lb. paracasei* subsp. *paracasei* CCM 1753 or the protective

culture were inoculated again into the finished cheese together with *G. candidum*. In this set of experiments, the initial cell densities of LAB were adjusted to > 10^7 CFU·g^{-1}.

Growth experiments and determination of microbial counts

The individual growth of *G. candidum* was studied in both cheeses at 5, 8, 10 and 12 °C. All co-culture experiments of the fungus and LAB were performed at 5 °C. Each growth experiment was performed in duplicate.

After appropriate sample dilution and cultivation, the numbers of each microorganism were determined and expressed as colony forming units per gram. *G. candidum* was enumerated on glucose-yeast extract-chloramphenicol agar (YGC, Merck). MRS agar (Biokar Diagnostics) was used for enumeration of *Lb. rhamnosus* GG and *Lb. paracasei* subsp. *paracasei*. The protective culture was enumerated on MRS agar supplemented with sodium lactate (1%, v/v).

Growth modelling and validation

The growth curves of *G. candidum* and LAB were fitted using the DMfit-model of Baranyi et al. [17], and growth parameters were estimated. The growth rates of *G. candidum* were used to externally validate the secondary growth model, which was developed in our previous work [18]. It is a cardinal model with inflection (CTMI) [19] describing the growth rate of *G. candidum* as a function of temperature in UHT milk. The following cardinal values were estimated: T_{min} = 1.41 °C, T_{max} = 35.34 °C, T_{opt} = 31.3 °C, μ_{opt} = 0.600 h^{-1} (T_{min}, T_{max}, T_{opt} symbols refer to the minimum, maximum and optimum temperature, respectively; μ_{opt} is the specific growth rate at the optimum temperature).

External validation of the secondary model was carried out for cottage cheese and the fresh cheese. The growth rates of *G. candidum* in both single culture and co-cultures were used for this validation. Validation data were obtained from these experiments in cottage cheese: *G. candidum* in single culture and in co-culture with *Lb. paracasei* subsp. *paracasei* at 5, 8, 10 and 12 °C. In the case of the fresh cheese, following experiments were used for validation: *G. candidum* in single culture and in co-culture with *Lb. paracasei* subsp. *paracasei* at 5, 8, 10 and 12 °C; *G. candidum* in co-culture with *Lb. rhamnosus* GG and the protective culture at 5 °C and 10 °C. To evaluate the performance of the model, bias (B_f) and accuracy (A_f) factors were calculated [20].

RESULTS AND DISCUSSION

Growth of *G. candidum* in cottage cheese and in fresh cheese prepared on a laboratory scale

The growth dynamics of *G. candidum* was determined in challenge tests with cottage cheese. The exponential growth of the fungus was observed at each storage temperature (Fig. 1). In spite of the lowest growth rate and the highest lag phase at 5 °C, the fungus was able to reach maximum numbers in approximately 12 days (Tab. 1). According to Görner and Valík [7], the sensory quality of acid-curd cheeses could be affected by the presence of yeasts and moulds at counts of 10^4–10^5 CFU·g^{-1}. The yeast-like fungus under study reached these numbers after 6–8 days of storage at 5 °C and only after 2–3 days at 12 °C. Guinee et al. [1] reported that the shelf-life of cottage cheese is limited to 2–3 weeks at the storage temperature of 5–7 °C. The challenge testing showed that cheese contamination and subsequent growth of *G. candidum* may lead to the spoilage before the recommended "use by date" despite adequate refrigeration. The following question arises from the results naturally: Which condition should be complied with in order to meet the shelf-life (28 days) declared by the producer at 5 °C?

The shelf-life of cottage cheese can be extended by the addition of chemical preservatives such as sorbic acid or potassium sorbate. Modified atmospheric packaging or dissolving of CO_2 in cheese seems to be promising for the purpose, too. On the other hand, a 'natural' way of extending the shelf-life of cottage cheese is the addition of protective cultures [1, 21]. The procedure of the manufacture of cottage cheese used in our experiments included the addition of the protective culture HOLDBAC YMB. This protective culture is active against yeasts, moulds and some heterofermentative lactic acid bacteria. It is believed that the antifungal activity of the protective culture is due to the production of some antifungal compounds, such as propionic acid, acetic acid, diacetyl and 2-pyrrolidone-5-carboxylic acid. However, the overall mechanisms underlying the antifungal action of protective cultures is not yet fully understood. It is due to complex and synergistic interactions between different low-molecular-weight compounds and likely cell-to-cell interactions. Because in cottage cheese the growth of *G. candidum* could be affected by the protective culture, we performed challenge tests in the fresh cheese produced under laboratory conditions (Fig. 1). The fungus was able to grow at a similar rate as in the cottage cheese and,

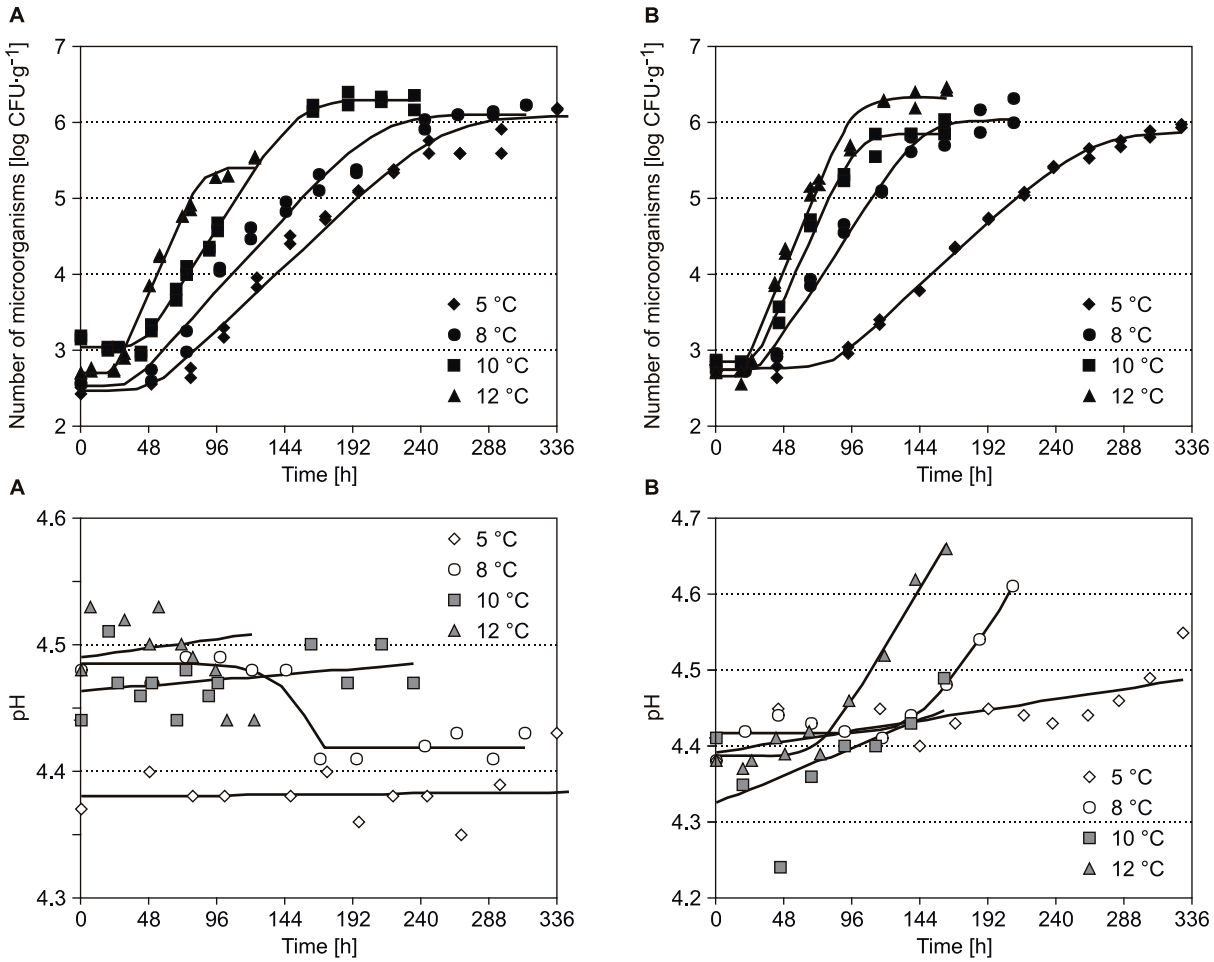

Fig. 1. Growth curves of *G. candidum* in cottage cheese and in fresh cheese prepared on a laboratory scale, and changes in pH.

A – cottage cheese; B – fresh cheese.

Tab. 1 Growth parameters of *G. candidum* in cottage cheese and in fresh cheese prepared on a laboratory scale.

T [°C]	Cottage cheese				Fresh cheese			
	μ [h^{-1}]	λ [h]	N_0 [log CFU·g^{-1}]	N_{max} [log CFU·g^{-1}]	μ [h^{-1}]	λ [h]	N_0 [log CFU·g^{-1}]	N_{max} [log CFU·g^{-1}]
5	0.041	50.6	2.47	6.10	0.039	79.0	2.76	5.88
8	0.048	35.8	2.54	6.11	0.067	31.7	2.75	6.04
10	0.068	43.5	3.04	6.31	0.095	27.3	2.85	5.86
12	0.100	23.2	2.70	5.41	0.106	16.8	2.66	6.32

T – temperature; μ – growth rate; λ – lag phase duration; N_0 – initial count; N_{max} – final count.

at 8, 10 and 12 °C, at a slightly higher rate with a shorter lag phase (Tab. 1). These results indicate that there was no or only weak effect of the protective culture against the growth of *G. candidum*. In order to confirm this assumption, in the next set of experiments the protective culture and *G. candidum* were inoculated simultaneously into the fresh cheese. The possible inhibitory effect of *Lb. rhamnosus* GG and *Lb. paracasei* subsp. *paracasei* CCM 1753 on the growth of *G. candidum* was also studied. *Lb. rhamnosus* GG was found to inhibit the growth of *G. candidum* in milk and the observed reduction in the growth rate was greatest at temperatures ranging from 10 °C to 15 °C [18,

22]. *Lb. paracasei* subsp. *paracasei* is a member of non-starter lactic acid bacteria (NSLAB) and some strains of this species are known to display antifungal activity.

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

When LAB were inoculated into the fresh cheese at the same time as *G. candidum*, their initial numbers always exceeded 6.5 log CFU·g^{-1}. The growth curve of the fungus obtained from each co-culture closely resembled the growth curve of a single culture. The growth rates of *G. candidum* reached similar values and the lag phases were shorter in comparison to the single culture (Tab. 2). The lowest lag phase was observed in co-culture with the protective culture.

None of the tested LAB strains was able to slow down the growth of *G. candidum*. This confirmed our previous assumption that the protective culture cannot control the growth of *G. candidum*. However, it should be taken into account that the antifungal activity of protective cultures depends also on the initial numbers of competitive bacteria. Schwenninger and Meile [23] studied the antifungal activity of three protective cultures consisting of *Lb. paracasei* subsp. *paracasei* strains SM20, SM29, SM63, each in combination with *Propionibacterium jensenii* SM11, against some spoilage yeasts in yoghurt. Yeasts reached numbers of 10^6–10^7 CFU·ml^{-1} if protective cultures were added into the yoghurt at concentrations of 10^7 CFU·ml^{-1}, while at 10^8 CFU·ml^{-1} no increase of viable yeasts was observed. A protective culture combining *Propionibacterium freudenreichii* subsp. *shermanii* SJ with *Lb. rhamnosus* LC705 was effective against yeasts and moulds at a level of 10^7 CFU·g^{-1}, whereas the lower level of 10^6 CFU·g^{-1} had no effect on the growth of *Rhodotorula rubra* RHO [24].

Growth curves obtained in the next set of co-culture experiments (the so-called pre-incubation experiments) are shown in Fig. 2. Despite the higher initial numbers of LAB in these co-cultures, again no inhibition of *G. candidum* growth was observed (Tab. 2). In co-culture with *Lb. paracasei* subsp. *paracasei*, the fungus reached cell counts of 10^4–10^5 CFU·g^{-1} after 7–10 days of storage at 5 °C. In co-cultures with *Lb. rhamnosus* GG and HOLDBAC YMB, it took *G. candidum* 7–9 days and 7–10 days, respectively, to reach such counts. These time periods were the same or prolonged only by one day in comparison with the single culture, which is insufficient from the practical point of view.

The growth of *G. candidum* in binary co-cultures with the same LAB strains was studied also at 10 °C. A partial growth inhibition of the fungus was observed at this temperature. From the tested bacteria, *Lb. rhamnosus* GG was the most effective in reducing the growth rate of *G. candidum* (42% decrease). In spite of this effect, the time needed for the fungus to reach a level of 10^4–10^5 CFU·g^{-1} was only half a day or one day longer than in the single culture. A stronger inhibition of the growth of *G. candidum* in co-culture with *Lb. rhamnosus* GG was observed in milk at 10 °C. However, a lower incubation temperature (5 °C) appeared to be unfavourable for the probiotic strain [18, 22].

External validation of the secondary model

At the lowest experimental temperature (5 °C), the model strongly underpredicted the growth rate of *G. candidum* in cottage cheese and also in the fresh cheese, as can be seen from Fig. 3. When this temperature was included in the validation

Tab. 2 Growth parameters of *G. candidum* and LAB in co-cultures.

Parameters	Without pre-incubation			With pre-incubation		
	Gc + LGG	Gc + LP	Gc + PC	Gc + LGG	Gc + LP	Gc + PC
μ_{Gc} [h^{-1}]	0.041	0.039	0.036	0.041	0.035	0.035
λ_{Gc} [h]	72.2	77.9	57.6	76.1	69.3	66.3
$N_{0,Gc}$ [log CFU·g^{-1}]	2.35	2.81	2.22	2.42	2.40	2.32
$N_{max,Gc}$ [log CFU·g^{-1}]	5.48	5.94	6.01	5.44	5.55	5.82
μ_{LAB} [h^{-1}]	–0.002	–0.014	–0.001	–0.004	0.0002	–0.0003
$N_{0,LAB}$ [log CFU·g^{-1}]	6.72	6.71	7.23	7.25	8.39	7.91
pH_{in}	4.28	4.38	4.30	4.31	4.30	4.32
pH_{end}	4.34	4.48	4.64	4.30	4.36	4.83

μ – growth rate; λ – lag phase duration; N_0 – initial count; N_{max} – final count; pH_{in}, pH_{end} – pH of the fresh cheese at the beginning and at the end of experiments; Gc – *G. candidum*; LGG – *Lb. rhamnosus* GG; LP – *Lb. paracasei* subsp. *paracasei* CCM 1753; PC – protective culture HOLDBAC YMB.

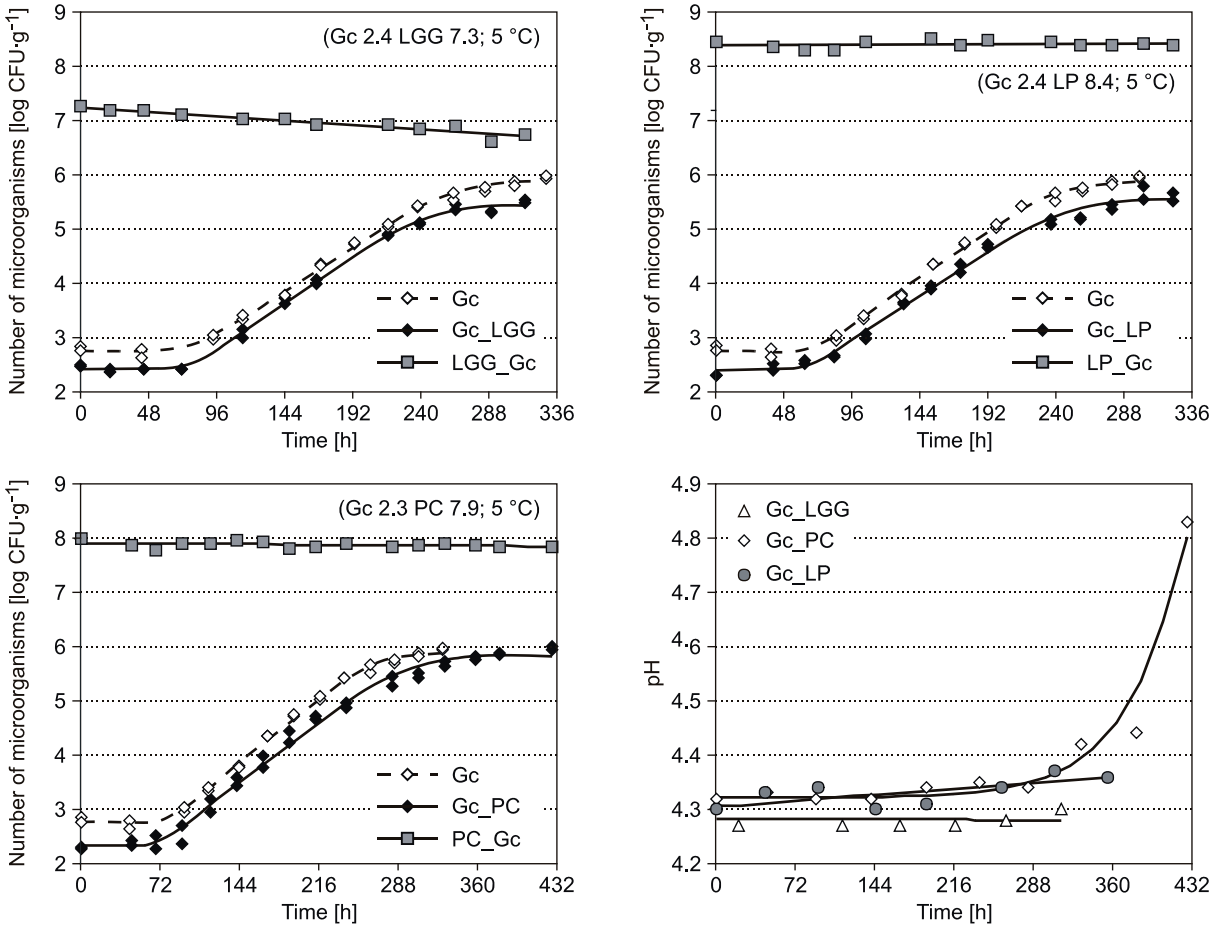

Fig. 2. Growth curves of *G. candidum* in co-culture with lactic acid bacteria and changes in pH.

The experiments were performed with pre-incubation of lactic acid bacteria in UHT milk used for the fresh cheese production. Gc – growth curve of *G. candidum* in pure culture; Gc_LGG, Gc_LP, Gc_PC – growth curves of *G. candidum* in co-cultures with lactic acid bacteria; LGG_Gc – growth curve of *Lb. rhamnosus* GG in co-culture, LP_Gc – growth curve of *Lb. paracasei* subsp. *paracasei* CCM 1753 in co-culture, PC_Gc – growth curve of protective culture HOLDBACTM YMB in co-culture.
Numbers in parentheses represent initial numbers of the respective microorganism expressed as logarithm of colony forming units per gram.

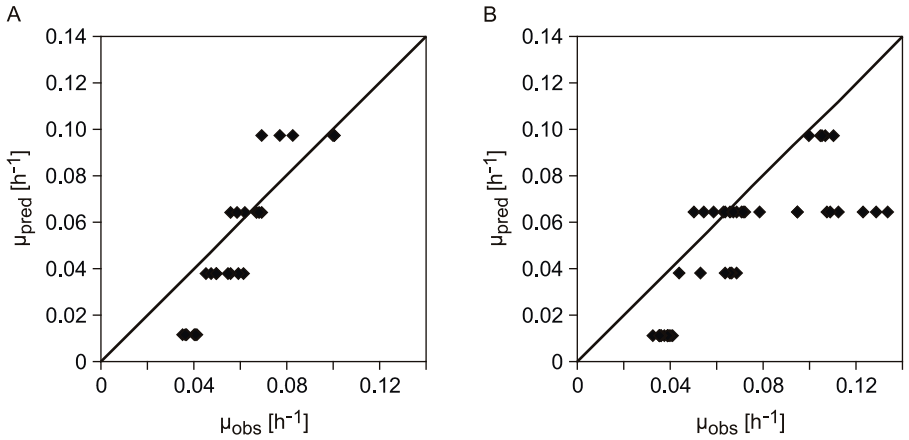

Fig. 3. Comparison of observed and predicted growth rates of *G. candidum* in the cottage cheese and in fresh cheese prepared on a laboratory scale.

A – cottage cheese; B – fresh cheese.
μ_{pred} – predicted growth rate; μ_{obs} – observed growth rate.

process, unacceptably high bias factors were obtained for both growth media (Tab. 3), as only the B_f value of 0.8–1.3 is considered acceptable for spoilage microorganisms in food [25]. Within the same temperature interval (5–12 °C), the A_f of 1.575 and 1.745 for the cottage cheese and fresh cheese, respectively, indicating that poor accuracy between the predictions and observations was obtained. If the temperature of 5 °C was withdrawn from the validation the resulting B_f values reached acceptable values (Tab. 3). Accuracy and bias factors in the cottage cheese were less than those calculated in the fresh cheese. The value of $A_f < 1.31$ indicated that the predictions had a deviation below 31 % compared to the observations. The comparable accuracy factors (1.26–1.38) were obtained in the study of BAERT et al. [26], when the validation was performed on a different medium. The external validation of kinetic models using real food matrices (maize grain, coffee beans and peanuts) led to acceptable results in most cases, but only if the optimal growth conditions were used. If the boundary conditions for the growth of studied aspergilli were used, a poor goodness of prediction was observed with unacceptably low B_f of 0.09–0.56, or high A_f (1.91–30.97; except of *A. parasiticus* on peanuts) [27]. In another study, the external validation performed on synthetic grape juice medium and on grape juice agar medium showed B_f and A_f in the range 1.01–1.06 and 1.11–1.29, respectively. In this case, a very good prediction was observed as the combined effect of temperature and water activity was modelled [28]. On the other hand, larger A_f values (1.50–1.61) were obtained when the same growth medium but different strains were used for validation of models describing the combined effect of temperature and water activity on the growth rate of *A. carbonarius* [29].

CONCLUSION

The fast growth of *G. candidum* was observed in the presence of the protective culture. In food matrices, where the conditions permit the growth of *G. candidum*, the predictive concept could be applied in order to minimize economic loss. For this purpose, the external validation of the predictive model describing the growth rate of *G. candidum* as a function of temperature was carried out. The model showed to be a reasonably good predictor, but only if the temperature had not dropped below 8 °C. On the other hand, the predicted growth was slower than that actually observed, which is fail-dangerous. The model is applicable in the cases of storage at higher refrigerator temperatures or if the storage temperature exceeded the required cooling limit.

Tab. 3. Mathematical indices used to validate the cardinal model with inflection for describing the effect of temperature on the growth rate of *G. candidum* in cottage cheese and in fresh cheese prepared on a laboratory scale.

Temperature interval	Cottage cheese		Fresh cheese	
	A_f	B_f	A_f	B_f
5–12 °C	1.575	1.458	1.745	1.707
8–12 °C	1.253	1.134	1.308	1.267

A_f – accuracy factor; B_f – bias factor.

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