Effect of media composition and CO_2 concentration on the growth and metabolism of *Lactobacillus acidophilus* NCFM

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Summary

A strain *Lactobacillus acidophilus* NCFM Howaru Dophilus (NCFM strain) is a probiotic bacterium with positive health effects proven by extensive research. This work deals with examination of media composition and CO_2 concentration effects on the growth and metabolism of *Lactobacillus* strains. Growth rate (*Gr*) of NCFM strain in non-supplemented de Man-Rogosa-Sharpe (MRS) broth was 0.348 log CFU·ml^{-1}·h^{-1}, lactic acid production rate (r_{LA}) was 2.07 g·l^{-1}·h^{-1} and the concentration of produced lactic acid (Δc_{LA}) reached 12.5 g·l^{-1}. Activity of NCFM strain in milk was significantly lower (*Gr* = 0.297 log CFU·ml^{-1}·h^{-1}, r_{LA} = 0.16 g·l^{-1}·h^{-1}, Δc_{LA} = 12.1 g·l^{-1}). Depending on the added substrate, *Gr* of NCFM strain was 0.295–0.398 log CFU·ml^{-1}·h^{-1} and 0.260–0.341 log CFU·ml^{-1}·h^{-1}, and the yield of lactic acid Δc_{LA} was 1.12–14.39 g·l^{-1} and 1.13–11.57 g·l^{-1}, in MRS broth and milk respectively. Results showed that the most significant stimulation was achieved in medium containing tryptone and the growth limiting factors were mainly free amino acids.

Keywords

Lactobacillus acidophilus NCFM; growth; metabolism; organic acids

Knowledge and ability to predict the behaviour of microorganisms in foods are required to ensure their safety and hygiene. Microbial growth and metabolism are affected by many intrinsic and extrinsic factors, including temperature and media composition. This gives the base of predictive microbiology, which is useful not regarding foodborne pathogens but also to predict the behaviour of technologically important microorganisms during food production and storage.

The bacterial culture *Lactobacillus acidophilus* NCFM Howaru Dophilus (NCFM strain) consists of a probiotic strain that was isolated from human gastrointestinal tract in year 1900. It is a homofermentative lactic acid bacterium that is able to proliferate well in milk due to a wide range of transport and fermentation systems [1]. Suitability of the NCFM strain for use in probiotic dairy products is supported by its good viability during storage at refrigeration temperatures [2].

Studies focused on probiotic and antimicrobial effects of *Lb. acidophilus* NCFM, which were collected in detail by Mančušková et al. [3], proved that the strain is able to survive in a human gastrointestinal tract and to inhibit pathogens by competing for nutrients and/or binding sites on epithelial cells. Production of specific and/or non-specific metabolites, like organic acids, diacetyl or bacteriocins, strengthens its antimicrobial effect and helps to prevent bacterial infection. Some research reported on immunomodulatory and tumour-suppressing activity of the NCFM strain [4–6]. The ability to strengthen the human immune system was confirmed by Scientific Opinion of the EFSA Panel on Dietetic Products, Nutrition and Allergies [7].

It was confirmed that the strain *Lb. acidophilus* NCFM is able to grow at a temperature range from 14.3 °C to 46.6 °C, with an optimum at 40.1–40.5 °C. Its maximal growth rate (*Gr*) in milk at an optimal temperature of 40.1 °C is 0.346 log CFU·ml^{-1}·h^{-1} [8]. However, *Lactobacillus* sp. is a large group whose members have different growth abilities under the same conditions and are not similar even to related species. For example, *Lb. rhamnosus* GG can grow in milk at temperatures from 2.7 °C to 52.0 °C, with a maximal growth rate 0.88 log CFU·ml^{-1}·h^{-1}

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at an optimal temperature of 44.4 °C [9]. *Lb. paracasei* ssp. *paracasei* 1753 is able to proliferate in MRS broth at 43 °C with a maximal growth rate 0.17–0.25 log CFU·ml^{-1}·h^{-1} [10]. A wide variability of growth dynamics among *Lactobacillus* spp. was documented by several studies [11–14], and also variability was observed among *Lb. acidophilus* strains [15, 16].

Despite the fact that the beneficial effects of NCFM strain on human health have been characterized by a large number of in vitro and in vivo studies, data describing its growth and metabolism dynamics are not generally available. That is why this work deals with the quantification of media composition and CO_2 concentration effects on the growth and metabolism of *Lb. acidophilus* NCFM Howaru Dophilus in a real and artificial growth medium.

MATERIALS AND METHODS

Microorganism

The strain *Lactobacillus acidophilus* NCFM Howaru Dophilus of Danisco (Copenhagen, Denmark) was provided for our study by Rajo (Bratislava, Slovakia). Identification and the monoculture composition of the culture were confirmed by Gram staining and microscopic examination, by API 50CHL test (BioMeriuéx, Marcy-l'Étoile, France) and by polymerase chain reaction (PCR) analysis of 16S rRNA gene according to Dubernet et al. [17] (data not shown).

Inoculation and cultivation conditions

The strain of *Lb. acidophilus* NCFM was kept in de Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) at (5 ± 1) °C. The standard suspension of the microorganism was prepared from a 24 h old culture of *Lb. acidophilus* NCFM grown in the MRS broth at 37 °C and 5 % CO_2 (CO_2 incubator; Binder, Tuttlingen, Germany). This culture was inoculated in concentration of approx. 10^3 CFU·ml^{-1} into 300 ml of pre-tempered ultra-pasteurized (UHT) milk (fat content 1.5 %, Rajo) or into MRS broth with or without added single substrates (glucose, fructose, lactose, saccharose, mannose, tryptone, cell-free supernatant of Fresco culture, cysteine hydrochloride). Three replicate samples of model media were incubated aerobically without shaking at (37 ± 0.5) °C. For determination of influence of atmosphere composition, the samples without added substrate were incubated without shaking at 15 % CO_2 and 37 °C.

Preparation of media

The solutions of saccharides (Mikrochem, Pezinok, Slovakia), tryptone (Biokar Diganostics) and L-cysteine hydrochloride (CysHCl; Lachema, Brno, Czech Republic) were prepared by dissolution of appropriate amounts of substrates in deionized water and sterilized by filtration through syringe microfilter with pore size 0.20 μm (Sarstedt, Nümbrecht, Germany).

For preparation of cell-free supernatant (CFS) of Fresco DVS 1010 culture (consisting of *Lactococcus lactis* ssp. *lactis*, *Lc. lactis* ssp. *cremoris* and *Streptococcus salivarius* ssp. *thermophilus*; produced by Christian Hansen, Hørsholm, Denmark; provided by Rajo), an overnight culture was prepared by inoculation of 1 ml of frozen Fresco culture into 100 ml of UHT milk and by subsequent cultivation aerobically without shaking at 30 °C for 24 h. The overnight culture was centrifuged (855 ×*g*, 20 min) and the supernatant was sterilized by filtration as described previously. The acid contribution of CFS to cultivation media was considered and deducted. The sterile solutions of substrates were added into sterile MRS broth (prepared following the manufacturer's instructions) and UHT milk in requisite concentrations.

Determination of *Lb. acidophilus* NCFM in milk and in MRS broth

In relevant time intervals, required volumes of the culture were withdrawn to determine the actual microorganism density by the dilution cultivation method on MRS agar (Biokar Diagnostics) according to ISO 20128:2006 [18]. Typical colonies were white, round to oblate and convex, and their morphology was occasionally confirmed by microscopic examination.

Determination of active acidity

In relevant time intervals, determination of pH values was performed using WTW Inolab 720 pH meter (WTW, Weilheim, Germany).

Determination of concentration of lactic acid and citric acid

Samples of media withdrawn in relevant time intervals were centrifuged (855 ×*g*, 10 min) and filtered through syringe microfilter with a pore size of 0.45 μm (Sarstedt). The samples were separated and analysed by EA 102 isotachophoretic analyser (Villa Labeco, Spišská Nová Ves, Slovakia) in its pre-separative column at direct current 250 mA using a conductivity detector and the electrophoretic system A5 (Comenius University, Bratislava, Slovakia): leading electrolyte HCl (1.0×10^{-2} mol·l^{-1}), aminocaproic acid

(1.0×10^{-2} mol·l^{-1}), hydroxy methyl cellulose (0.1 % w/w), pH 4.25; terminating electrolyte: caproic acid (5.0×10^{-3} mol·l^{-1}), Tris(hydroxymethyl) aminomethane (5.0×10^{-3} mol·l^{-1}), pH 4.5–5.0.

Detection of diacetyl

Presence of diacetyl formed by transformation of citrates was detected by Voges-Proskauer test [19].

Fitting the growth curves and calculating the growth parameters

The growth data (lag phase λ, growth rate *Gr*, rate of lactic acid production r_{LA}, active acidity rate r_{pH}, lactic acid production Δc_{LA} and citric acid production Δc_{CA}), curves and parameters of the strain under study were analysed, fitted and calculated, respectively, using the mechanistic modelling technique of Baranyi et al. [20], incorporated in the DMFit tools kindly provided by Dr. J. Baranyi (Institute of Food Research, Norwich, United Kingdom).

Statistical analyses

Each experiment was performed in three separate trials. Results are represented by means of values with their standard deviations. Statistical analyses were carried out using Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA). Parametric data were treated by independent two-samples Student *t*-test (unequal variances) and confirmed by ANOVA test. Non-parametric data were treated by Kruskal-Wallis test. All the tests used 95% confidence intervals.

RESULTS AND DISCUSSION

Growth and metabolism dynamics in MRS broth and UHT milk

Growth and metabolism dynamics of NCFM strain at 37 °C, depending on basic media composition, were evaluated as an increase in microbial counts and as production of organic acids, depending on cultivation time (Fig. 1). Only rapid

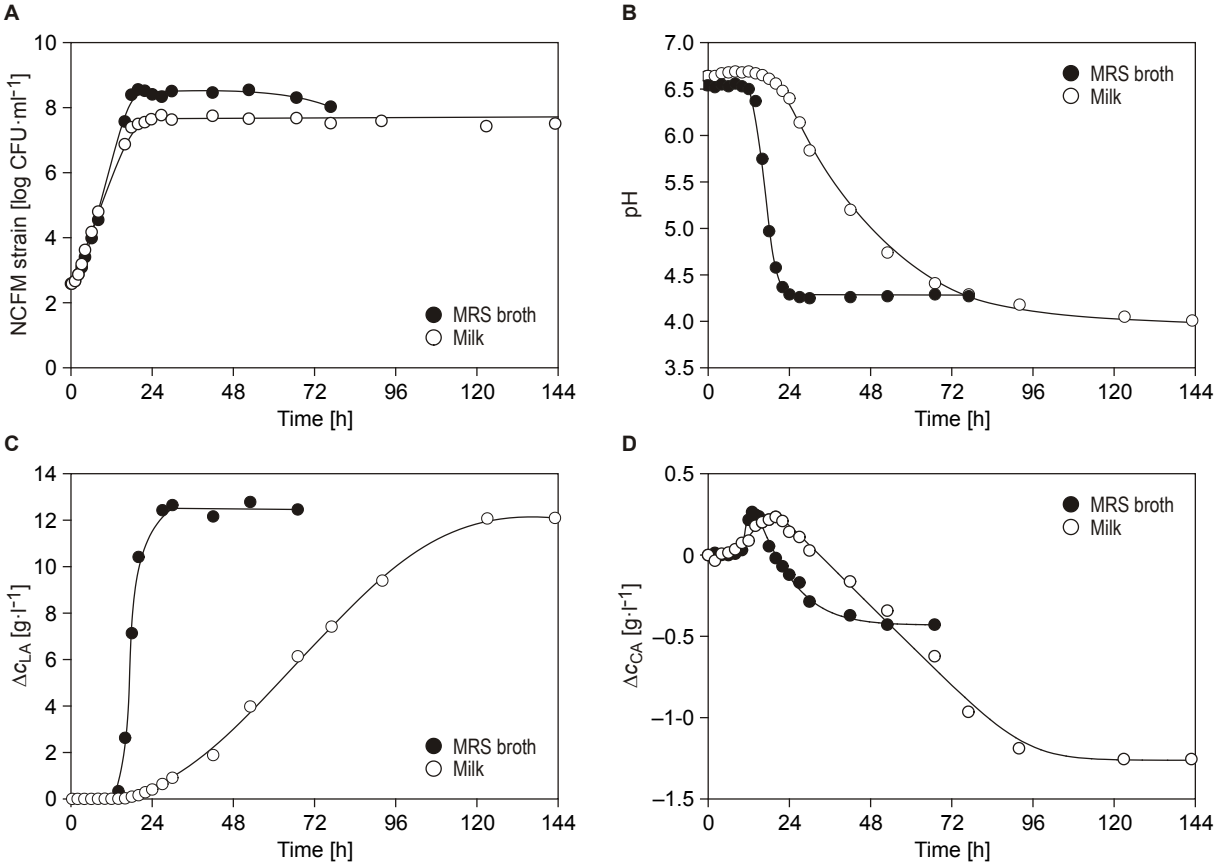

Fig. 1. Growth and metabolism dynamics of *Lactobacillus acidophilus* NCFM in MRS broth and in milk.

A – counts of *Lactobacillus a* NCFM, B – pH value of media, C – change of lactic acid concentration, D – change of citric acid concentration.

production of lactic acid and low production of citric acid, the latter being followed by its subsequent consumption, were observed during cultivation. The concentrations of other acids was still under the limit of detection (*LOD*) during the entire cultivation time in all experiments. The *LOD*s for organic acids were as follows (expressed as moles per litre): lactic acid 0.022×10^{-3}, acetic acid 0.043×10^{-3}, propionic acid 0.030×10^{-3}, citric acid 0.028×10^{-3}, malic acid 0.020×10^{-3}, succinic acid 0.047×10^{-3}, aspartic acid 0.045×10^{-3}, tartaric acid 0.015×10^{-3} and 4-hydroxyphenylacetic acid 0.026×10^{-3}. Based on these results, the strain *Lb. acidophilus* NCFM can be assigned to obligatory homofermentative lactobacilli, which is in agreement with literature [21–23].

Intensive growth of *Lb. acidophilus* NCFM at 37 °C was observed during cultivation in MRS broth (Gr = 0.348 log CFU·ml^{-1}·h^{-1}; λ = 1.6 h). The NCFM strain started to produce lactic acid 14.7 h after its inoculation into MRS broth and the production was the most intensive (r_{LA} = 2.07 g·l^{-1}·h^{-1}) at the end of the exponential growth phase. Maximum yield of lactic acid was Δc_{LA} = 12.5 g·l^{-1} after 24 h of incubation. Growth dynamics in milk were slower compared to those in MRS broth (Gr = 0.297 log CFU·ml^{-1}·h^{-1}; λ = 1.7 h), as can be seen in Fig. 1A. Although maximum yields of lactic acid were similar in milk and in MRS broth (production of lactic acid in milk after 120 h was Δc_{LA} = 12.1 g·l^{-1}), the metabolism of NCFM strain was also slower and the rate of lactic acid production in milk (r_{LA} = 0.16 g·l^{-1}·h^{-1}) was thirteen times lower than the production rate in MRS broth (Fig. 1C). Increase in organic acids concentration was reflected by the corresponding rate of active acidity decrease in both media. Rates of pH decrease reached r_{pH} = –0.34 h^{-1} and r_{pH} = –0.13 h^{-1} in MRS broth and milk, respectively (Fig. 1B).

Statistical analysis showed that MRS broth is more suitable for growth and metabolism of NCFM strain, compared to milk. Evaluation of data from both media using Kruskal-Wallis test revealed a significant difference ($P > 0.05$) of growth rates and lactic acid production in favour of MRS broth.

By isotachophoretic analysis, a temporary increase in concentration of citric acid was observed (Fig. 1D), the maximum production achieving approx. 0.24 g·l^{-1} in both cultivation media. After the peak, the concentration of citrate was gradually reduced until complete exhaustion. This could be caused by transformation of citrates into other metabolites, including sensorically active secondary metabolites. One of possible products could be diacetyl, which was also determined by a Voges-Proskauer test. Also ØSTLIE et al. [24] observed that some lactobacilli produced higher amounts of diacetyl at presence of citrate in the cultivation media.

Effects on growth

Besides monitoring the culture growth and the production or consumption of organic acids in time, effects of media composition and CO_2 concentration on growth and metabolic activity of *Lb. acidophilus* NCFM were evaluated as well. Considering the high heterogenity of microorganisms, composition of an ideal cultivation medium may vary. Its suitability for a specific organism depends on contents of vitamins, biogenic elements and carbon, nitrogen and sulphur sources. Therefore, we performed a series of experiments to analyse changes in growth and metabolic parameters in real and artificial media supplemented with substrates listed previously (Tab. 1, Tab. 2). Additions did not affect water activity values, which were higher than 0.998 in all cases. Therefore, the effect of reduced water activity on NFCM strain could be excluded.

Generally, the NCFM strain showed better growth in MRS broth compared to milk. Growth rate of the strain under study in MRS medium ranged from 0.295 log CFU·ml^{-1}·h^{-1} to 0.398 log CFU·ml^{-1}·h^{-1}, depending on the added substrate. Lag phase duration was changing around the average value of 1.89 h ± 0.37 h (coefficient of variation CV = 19.3 %).

Similarly to cultivation experiments in MRS broth with addition of different substrates, average lag phase duration of NCFM strain in supplemented milks was 1.76 h ± 0.51 h (CV = 29.3 %). However, differences in media composition were reflected by significant differences ($P < 0.05$ in t-test and ANOVA test) in growth rate of the strain (growth rates ranged from 0.260 log CFU·ml^{-1}·h^{-1} to 0.341 log CFU·ml^{-1}·h^{-1}). Independent of the added substrates, growth dynamics of *Lb. acidophilus* NCFM were slower in milk than in unsupplemented MRS broth.

Addition of carbohydrates had no effect on growth of the strain in a desired manner. Growth rates of NCFM strain in all saccharide-supplemented media were similar or lower than the growth rate in the unsupplemented medium. It ranged from 0.295 log CFU·ml^{-1}·h^{-1} to 0.346 log CFU·ml^{-1}·h^{-1} in MRS broth, and from 0.260 log CFU·ml^{-1}·h^{-1} to 0.292 log CFU·ml^{-1}·h^{-1} in milk. While the addition of saccharose slowed down proliferation by 12–15 %, addition of fructose to milk was reflected by reduction of the

Tab. 1. Growth parameters and changes of lactic acid and citric acid concentrations during cultivation of *Lb. acidophilus* NCFM in MRS broth.

Substrate	*Gr* [log CFU·ml^{-1}·h^{-1}]	λ [h]	pH	Δc_{LA} [g·l^{-1}]	Δc_{CA} [g·l^{-1}]
Non-supplemented	0.348 ± 0.004	1.57 ± 0.01	4.26	12.44 ± 0.37	0.83 ± 0.03
Glucose 2 g·l^{-1}	0.316 ± 0.011 *	2.61 ± 0.01 *	4.15	11.75 ± 0.27	0.87 ± 0.04
Fructose 2 g·l^{-1}	0.336 ± 0.018	2.16 ± 0.94 *	4.06	14.39 ± 0.26 *	1.18 ± 0.06 *
Maltose 2 g·l^{-1}	0.341 ± 0.004	2.02 ± 0.21 *	4.12	11.13 ± 0.39	0.80 ± 0.04
Saccharose 2 g·l^{-1}	0.295 ± 0.020 *	1.64 ± 0.96	4.02	12.41 ± 0.37	0.88 ± 0.03
Lactose 5 g·l^{-1}	0.346 ± 0.004	1.38 ± 0.19	4.09	12.39 ± 0.22	0.87 ± 0.04
Tryptone 5 g·l^{-1}	0.387 ± 0.009 *	1.57 ± 0.28	4.08	13.89 ± 0.47	0.71 ± 0.07 *
CFS 5% (v/v)	0.384 ± 0.006 *	1.64 ± 0.11	3.99	11.60 ± 0.27	0.88 ± 0.04
CFS 10% (v/v)	0.363 ± 0.002	1.82 ± 0.13 *	4.04	13.06 ± 0.34	0.99 ± 0.05 *
CysHCl 5 g·l^{-1}	0.398 ± 0.001 *	2.36 ± 0.10 *	4.02	14.01 ± 0.37 *	0.70 ± 0.04 *
CO_2 15%	0.342 ± 0.005	1.75 ± 0.23 *	4.08	1.12 ± 0.13 *	1.02 ± 0.05 *

Values are expressed as mean ± standard deviation. Values determined in supplemented media marked with an asterisk (*) are significantly different ($P > 0.05$ in *t*-test and ANOVA test) from values determined in non-supplemented media.
Gr – growth rate of *Lb. acidophilus* NCFM, λ – lag phase, pH – pH value at the end of exponential growth phase, Δc_{LA} – change of lactic acid concentration, Δc_{CA} – change of citric acid concentration, CFS – cell-free supernatant of Fresco culture, CysHCl – cysteine hydrochloride.

Tab. 2. Growth parameters and changes of lactic acid and citric acid concentrations during cultivation of *Lb. acidophilus* NCFM in milk.

Substrate	*Gr* [log CFU·ml^{-1}·h^{-1}]	λ [h]	pH	Δc_{LA} [g·l^{-1}]	Δc_{CA} [g·l^{-1}]
Non-supplemented	0.297 ± 0.003	1.68 ± 0.10	5.84	0.96 ± 0.01	0.03 ± 0.00
Glucose 2 g·l^{-1}	0.291 ± 0.005	2.86 ± 0.70 *	4.57	6.76 ± 0.07 *	–0.68 ± 0.01 *
Fructose 2 g·l^{-1}	0.282 ± 0.006 *	1.18 ± 0.23 *	4.18	1.98 ± 0.02 *	–0.72 ± 0.02 *
Maltose 2 g·l^{-1}	0.294 ± 0.003	1.83 ± 0.08	5.44	2.14 ± 0.03 *	–0.21 ± 0.01
Saccharose 2 g·l^{-1}	0.260 ± 0.010 *	1.46 ± 0.34 *	4.33	8.09 ± 0.08 *	1.70 ± 0.05 *
Lactose 5 g·l^{-1}	0.292 ± 0.002	1.32 ± 0.08 *	5.18	1.89 ± 0.02 *	–0.29 ± 0.01
Tryptone 5 g·l^{-1}	0.326 ± 0.003 *	< 1 *	4.15	11.57 ± 0.12 *	0.45 ± 0.00 *
CFS 5% (v/v)	0.326 ± 0.002 *	1.28 ± 0.10 *	4.59	6.38 ± 0.08 *	–0.73 ± 0.02 *
CFS 10% (v/v)	0.284 ± 0.001 *	1.63 ± 0.05	4.80	5.64 ± 0.07 *	0.48 ± 0.03 *
CysHCl 5 g·l^{-1}	0.284 ± 0.013 *	2.03 ± 0.74 *	5.16	2.25 ± 0.02 *	–0.26 ± 0.00
CO_2 15%	0.341 ± 0.004 *	2.24 ± 0.07 *	5.69	1.13 ± 0.01	0.45 ± 0.00 *

Values are expressed as mean ± standard deviation. Values determined in supplemented media marked with an asterisk (*) are significantly different ($P > 0.05$ in *t*-test and ANOVA test) from values determined in non-supplemented media.
Gr – growth rate of *Lb. acidophilus* NCFM, λ – lag phase, pH – pH value at the end of exponential growth phase, Δc_{LA} – change of lactic acid concentration, Δc_{CA} – change of citric acid concentration, CFS – cell-free supernatant of Fresco culture, CysHCl – cysteine hydrochloride.

lag phase duration by almost 30%. This phenomenon may be related to the smaller number of reaction steps, which are necessary for utilization of fructose during glycolysis. WISSELINK et al. [25] and CARVALHEIRO et al. [26] reported that, under adequate oxygen availability conditions, glucose can be used as an energy and carbon source, and fructose as an electron acceptor by various strains of lactobacilli. Under these conditions, the net gain of ATP is 2 mol of ATP per 1 mol of fermented fructose. This can lead to acceleration of metabolism and to reduction of lag phase duration, in particular in heterofermentative lactobacilli.

Combination of a starter culture with probiotic bacteria, with the aim to achieve complementary features of fermented products, is generally applied in industrial practice. Different combinations of starter lactic and probiotic cultures allow

the production of dairy products with desired technological characteristics, and potential nutritional and health benefits [27, 28]. In this study, CFS of Fresco starter culture was added into cultivation media to support the growth and metabolism of the probiotic bacterium *Lb. acidophilus* NCFM. Growth rate of the probiotic strain in MRS broth reached values of 0.384 log CFU·ml^{-1}·h^{-1} and 0.363 log CFU·ml^{-1}·h^{-1} (a 10.3% and 4.3% increase in comparison to reference media) in the presence of 5% and 10% addition of Fresco CFS, respectively. Results of cultivation experiments in CFS-supplemented milks followed the same trend. The stronger stimulation of growth was observed in medium with 5% of CFS addition. Rates of proliferation reached 0.326 log CFU·ml^{-1}·h^{-1} and 0.284 log CFU·ml^{-1}·h^{-1} in milks with 5% and 10% CFS added, respectively. Effect of Fresco CFS on lag phase duration was indeterminate.

Considering the high resistance of NCFM strain to lactic acid (data not shown), utilization of free nutrients, which are present in the Fresco CFS in majority, and a subsequent necessity of adaptation to complex nutrients could be a reason for the observed insufficient stimulation of 10% CFS supplementation on NCFM strain's growth dynamics. On the other hand, a possible reason for the higher growth rate in MRS broth supplemented by CFS could be due to the presence of free amino acids formed from milk casein by Fresco culture. This assumption is supported also by the highest growth rates (0.387 log CFU·ml^{-1}·h^{-1} and 0.326 log CFU·ml^{-1}·h^{-1} in MRS broth and milk, respectively), which were observed during NCFM cultivation in the presence of tryptone. Furthermore, the shortest lag phase of NCFM strain was determined in milk with tryptone addition, which was less than 1 h.

Several authors [29–32] observed limitation of growth of lactic acid bacteria as a result of biosynthesis of non-essential amino acids, such as tryptophan, and stimulation of growth of lactobacilli by tryptophan. The higher content of tryptophan (1% w/w) in tryptone used for supplementation of media could be responsible for stimulation of NCFM strain's growth.

The redox potential (E_h) is another environmental factor that has a considerable effect on the growth and metabolism of microorganisms. E_h value depends on the contents ratio of substances that are electron acceptors or donors. The addition of reducing agents (e.g. cysteine hydrochloride) and/or reduction of the oxygen partial pressure reduce the redox potential of media [22]. According to calculation in the study by Savescu's [33], the reducing agent decreases E_h levels in milk from 220–290 mV to about 0 mV. Lower E_h levels meet the growth conditions of facultative anaerobic microorganisms, including *Lactobacillus* spp. [34]. Considering that lower E_h levels stimulate the growth and metabolism of lactobacilli, the addition of cysteine hydrochloride into milk and MRS broth, to a final concentration of 5 g·l^{-1}, led to an increase in the growth rate of NCFM strain by 10% and 15%, respectively. This is consistent with results of Dave and Shah [35], who observed a reduction of time needed for complete fermentation of yoghurts by *Lb. acidophilus* in samples supplemented by cysteine hydrochloride.

In contrast to the addition of CysHCl, the indeterminate effect of 15% CO_2 in atmosphere on the growth of NCFM strain was observed. Although the growth rate of the strain in milk was higher by about 15% at cultivation in atmosphere with 15% CO_2 compared to the reference experiment, the change of atmosphere composition did not affect the growth rate of the strain in MRS broth significantly ($P > 0.05$ in *t*-test and ANOVA test). The lag phase durations were higher in all experiments with reduced redox potential compared to reference experiments.

Effect on metabolism

Effect of CO_2 concentration and media composition on metabolism of *Lb. acidophilus* NCFM was expressed as dependence of lactic acid and citric acid concentrations on the added substrate. Cultivation media were sampled in the early stationary phase of growth after 27 h of incubation at (37 ± 0.5) °C and analysed by isotachophoresis. As shown in Fig. 1C, lactic acid production in MRS broth was very intensive and reached its peak at the time of sampling. The concentration of lactic acid was affected by supplements in MRS broth only to a small extent. This was also expressed by small variation of organic acids concentrations. The concentration of lactic acid in cultures grown in supplemented MRS broth reached the value of $\Delta c_{LA} = (12.74 \pm 1.17)$ g·l^{-1}, with $CV = 9.2\%$ and the concentration of citric acid was $\Delta c_{CA} = 0.89 \pm 0.15$ g·l^{-1} ($CV = 16.3\%$). On the other hand, differences in organic acid production during cultivation of *Lb. acidophilus* NCFM in UHT milk were notable (Tab. 2).

Simple saccharides enter the catabolic pathway of carbohydrates directly without transformation, and lactose is present in milk in excess (considering the rate of lactose consumption by NCFM strain). Therefore, no significant effect of glucose, fructose or lactose on acid-generating activity of the NCFM strain was observed.

On the other hand, addition of disaccharides

(maltose, saccharose) led to a three-fold increase of lactic acid concentration compared to unsupplemented media. Remarkably high citric acid level was observed in milk with saccharose addition (Δc_{CA} = 1.70 g·l^{-1}). This can be related to unusually low concentration of citrates in milk used for this experiment (up to 67% below the average value for milk), which was detected in a milk sample taken at inoculation time.

The fastest decrease of active acidity was observed in tryptone-supplemented milk. Isotachophoretic analysis showed that pH drop was related to a rapid increase of lactic acid concentration in milk with tryptone addition, which was about six times higher compared to unsupplemented milk. Tab. 1 shows that lactic acid level in milk with tryptone addition (Δc_{LA} = 11.57 g·l^{-1}) was the highest among all milk substrates. However, the rate of utilization of citric acid was reduced at the expense of higher lactic acid production. Yield of citrates at the time of sampling was positive (Δc_{CA} = 0.45 g·l^{-1}) in milk supplemented with tryptone, which was different from the majority of samples.

Zhao et al. [36] reported a reduction of time required for full milk fermentation during yoghurt production and enhancement of acid-generating activity of starter culture (*S. thermophilus, Lb. delbrueckii*) and probiotic culture (*Lb. acidophilus*) under similar conditions. Results suggest that casein hydrolysate can stimulate metabolism of probiotic lactic acid bacteria. As discussed above, stimulation of the NCFM strain's metabolism may result from the high contents of tryptophan in tryptone.

Fresco starter culture produces, inter alia, a large amount of lactic acid and the final CFS contained 15.0 g·l^{-1} of the metabolite. Although a the concentration of this substance was high, it did not inhibit growth of the probiotic strain. The addition of Fresco CFS into cultivation media led to a three-fold increase in lactic acid produced by NCFM strain at both added concentrations of CFS (Δc_{LA} = 5.16 g·l^{-1} and Δc_{LA} = 4.59 g·l^{-1} in 5% and 10% CFS-supplemented milk, respectively). Similar to data obtained from growth dynamics analysis, different concentrations of Fresco culture metabolites did not affect metabolic rates of the probiotic strain differently. It can even be assumed that the NCFM strain's metabolism of complex substances started slower in milk with 10% CFS, as illustrated by the positive yield of citric acid in this medium (Δc_{CA} = 0.48 g·l^{-1}) and a delayed drop of pH value. Data from experiments with CFS-supplemented media suggest that the higher addition of supplement need not result in higher stimulation of growth and metabolism of the NCFM strain.

Similar to growth dynamics data determined in CysHCl-supplemented media, the addition of the reducing agent into milk did not result in desired metabolic stimulation. Concentration of lactic acid in time of sampling was only two-times higher (Δc_{LA} = 2.25 g·l^{-1}) than in the reference experiment, and the pH value did not fall below 5.0.

The reduction of redox potential by atmosphere modification did not stimulate metabolism of the NCFM strain efficiently. Lactic acid concentration in 27th hour of cultivation was around Δc_{LA} = 1.1 g·l^{-1} in both media incubated in atmosphere with 15% of CO_2, and citric acid yielded Δc_{CA} = 1.02 g·l^{-1} and Δc_{CA} = 0.45 g·l^{-1} in MRS broth and milk, respectively. According to Hong and Pyun [37] and Singh et al. [38], slower metabolic activity of NCFM strain under anaerobic conditions can be related to the binding of CO_2 to specific enzymes or proteins, and consequent inhibition of metabolic pathways.

CONCLUSIONS

Based on the results, it can be concluded that the probiotic strain grew and metabolized slowly in milk. A delayed drop of pH value during the cultivation experiments in this medium, compared with experiments in MRS broth, was observed not only because of buffering capacity of the medium but mainly because of slower production of lactic acid in milk. Results showed that the artificial cultivation medium was more suitable for growth and metabolism of *Lactobacillus acidophilus* NCFM Howaru Dophilus than the milk medium.

While the addition of various saccharides did not provide a considerable stimulation of bacterial metabolism ($P > 0.05$ in *t*-test and ANOVA test), the addition of casein hydrolysate and metabolic products of Fresco culture affected NCFM strain's behaviour significantly ($P < 0.05$ in *t*-test and ANOVA test). Results indicate that carbohydrates are not the limiting factors for the strain growth. On the other hand, free amino acids are strongly required. Results confirmed that media optimization can lead to stimulation of growth and/or production of some desirable metabolites. Data can serve for optimization of technological processes during production of probiotic foods and dietary supplements enriched by *Lactobacillus acidophilus* NCFM, but analysis of the effect of added substances on organoleptic properties of the final product should precede.

In the context of predictive microbiology, the obtained results can serve to predict the time

needed to reach necessary cell densities and/or to predict changes in metabolism of the strain. Both of these are strongly dependent on cultivation conditions.

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