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From "Viili" Towards "Termoviili", a Novel Type of Fermented Milk: Characterization of Growth Conditions and Factors for a Co-culture of *Lactobacillus delbrueckii* and *Geotrichum candidum*

Tapani Alatossava*, Ruojie Li and Patricia Munsch-Alatossava
Department of Food and Environmental Sciences, University of Helsinki, Finland

***Address for Correspondence**
Tapani Alatossava, Department of Food and Environmental Sciences, P.O. Box 66, FI-00014 University of Helsinki, Helsinki, Finland, Tel: +358 9 191 58312; Fax: +358 9 191 58460; Email: tapani.alatossava@helsinki.fi

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Abstract

The traditional Northern fermented milk product "Viili" is based on the use of a starter comprising both mesophilic lactic acid bacteria (LAB) and *Geotricum candidum* mold strains for milk fermentation at 18 to 20°C for about 20 hours. The goal of the present study was to investigate whether there is a microbiological basis for the conception of a novel type of fermented milk product "Termoviili", which would be a hybrid between "Viili" and yogurt, because of the use of thermophilic *Lactobacillus delbrueckii* instead of mesophilic LAB as a starter component. Accordingly some critical growing conditions and factors that support a co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV strains were determined and further compared to a yogurt starter like co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *Streptococcus thermophilus* T101 strains. Both *Lb. delbrueckii* and *G. candidum* could grow at a temperature range of 30-34°C, and optimally at 30°C. At this temperature, the co-culture of *Lb. delbrueckii* and *G. candidum* showed similar synergistic interactions, like the yogurt starter co-culture, when growth occurred in a milk base, which had not been subjected to strong heat treatment like autoclaving at 121°C for 10 minutes. Formic acid, at a concentration of 7.35 mM, stimulated the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808, likewise when *G. candidum* VV or *Str. themophilus* T101 were grown in a co-culture with *Lb. delbrueckii* ssp. *lactis* ATCC 15808. These results suggest that *G. candidum*, like *Str. thermophilus,* produces formic acid which stimulates the growth of *Lb. delbrueckii*. Furthermore, a strong heat treatment of the milk base generated the same stimulatory effect on *Lb. delbrueckii,* as observed when *Lb. delbrueckii* was grown in the presence of 7.35 mM formate. The results obtained demonstrate that a co-culture of *Lb. delbrueckii* and *G. candidum* is based on a synergistic association, and accordingly these two species could be applied together as a starter in the manufacture of "Termoviili", a novel type of fermented milk product with potential new sensory and nutritional properties.

Introduction

Fermented milks have been manufactured and consumed for a long time in human history. Various starters including lactic acid bacteria (LAB) and other bacteria, yeasts and molds are key determinants for the production of fermented milk products with different tastes and flavors [1,2]. Fermented milks are beneficial to human health, conditioning the intestine environment, lowering the blood pressure, and reducing the risks of bladder cancer and colon cancer [3-8]. Nowadays, the increasing consumption of fermented milks offers a potential market for novel fermented milk products [9].

Globally among the commercial fermented milk products, yogurt is the most popular product. Yogurt belongs to the thermophilic homolactic (acid) fermentation type of fermented milks since two thermophilic LAB species, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus,* are used together as the components of a yogurt starter [2]. An associative growth (protocooperation) has been observed during fermentation between these two bacterial species. Based on the literature, growths factors such as carbon dioxide (CO_2) and formic acid produced by *Streptococcus thermophilus,* and particular amino acids especially valine provided by *Lactobacillus delbrueckii* ssp. *bulgaricus* proteolysis, are responsible for the observed synergistic effects [10-15]. Furthermore, it has been reported that formic acid is produced as a degradation product of lactose when milk bases are subjected to severe heat treatments [2].

Geotrichum candidum is an anamorphic and filamentous yeast-like mold [16]. It can grow at temperatures ranging from 5 to 38°C, with an optimal growth at around 25°C. Growth occurs within a large pH interval from 3 to 11, with an optimal interval at 5.0-5.5. Besides the long lag phase, the generation time of *G. candidum* is 66 min in liquid culture at 30°C, being one of the shortest among eukaryotes, with final counts (tfu, thallus forming units) lower than 10^6 tfu/g [17,18]. *G. candidum* is usually involved in the ripening of various mold cheeses. Among various types of fermented milks "Viili", a traditional fermented milk product mainly produced and consumed in Finland and in Sweden, is unique as its production is based on the use of a "Viili" starter containing both mesophilic LAB and *G. candidum* mold strains [2,19,20]. Unfortunately during the last 20 years the consumption of traditional "Viili" has declined to the benefit of a higher consumption of yogurt in Finland.

In the present study, we investigated whether there was a microbiological basis for the conception of a novel type of fermented

milk, which would be a "hybrid" product between "Viili" and yogurt. In this kind of fermented milk, the mesophilic LAB of traditional "Viili" starter strains would be replaced by a thermophilic *Lactobacillus delbrueckii* strain or strains. In other words, the study consisted in examining whether the *Streptococcus thermophilus* species of the yogurt starter could be replaced by the *G. candidum* species to achieve successful milk fermentation.

Materials and Methods

Bacteriological Media, Milk Bases and Chemicals

Skim milk powder, Yeast extract, M17 broth, MRS broth, sodium bicarbonate, sodium formate and disodium carbonate were purchased from Sigma-Aldrich (St. Louis, USA). M17 agar and yeast extract glucose chloramphenicol agar (YGC agar) were supplied by Merck (Darmstadt, Germany). MRS agar was from Lab M Ltd. (Lancashire, UK). The features of the milk bases are listed in Table 1.

Microorganisms, their Culture, and their Enumeration Procedures

Stock cultures of strains *Lactobacillus delbrueckii* ssp. *lactis* ATCC 15808, *Streptococcus thermophilus* T101 (Valio Ltd., Finland) and *Geotrichum candidum* VV (isolated from the "Viili" product manufactured by Valio Ltd., Finland) were preserved at -20°C.

Overnight cultures of strains ATCC 15808, T101 and VV were prepared in MRS, M17 and YGC broths, respectively. Strains ATCC 15808 and T101 were grown at 37°C, and strain VV at 30°C. The cell pellets were obtained by centrifugation of the cultures at 4000 rpm/5min followed by washing of the culture pellets twice with 0.85% NaCl solution. The cell pellets were subsequently resuspended into 2 mL 0.85% NaCl solution. Inoculation volumes of 100 µL for both ATCC 15808 and T101, and 150 µL for VV cell suspensions were transferred to 10 mL of the growth medium base. Both YGC and MRS broths were tested for the growth studies of *G. candidum* VV. Cultures were incubated at the considered temperature ranges. Bacterial growth was evaluated by (i) the enumeration of viable cells on agar plates, and (ii) the pH measurement of the cultures. The platings for the strains ATCC 15808 and T101 were performed on MRS-pH 5.4 agar incubated anaerobically, and on M17 agar incubated aerobically at 37°C, respectively. Anaerobic incubation conditions occurred in jars with AnaeroGen™ sachets (Oxoid Ltd., Hants, UK). The growth of *G. candidum* VV was determined on YGC agar under aerobic conditions at 30°C.

Results

Determination of an Optimal Growth Temperature for the Co-Culture of *Lb. delbrueckii ssp. lactis* ATCC 15808 and *G. candidum* VV

To investigate synergistic type of interactions, an overlap of the temperature ranges allowing the growth of both components of a co-culture is of a crucial importance. Accordingly, the growths of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV were followed separately in MRS broth during 48 hours, in a temperature range from 30°C to 42°C. Two initial inoculation levels (high and low) were included in both cases. The results of these growth tests have been summarized in Figure 1. In all conditions, the incubation time of 24 hours was sufficient to reach the maximal level of viable cells in the culture. Especially with high initial inoculation levels, significant losses of *G. candidum* cell viability were observed at 37°C and 42°C (Figure 1A). The optimal growth temperature for *G. candidum* VV was 30°C, the lowest among the tested temperatures. Above 34°C, the growth was seriously if not completely prevented (Figure 1A and B). The thermophilic *Lb. delbrueckii* ssp. *lactis* ATCC 15808 could grow equally well at any of the tested incubation temperatures (Figure 1C and D). The best conditions for cell survival could be reached at 30°C and 32°C when a high initial inoculation level was tested (Figure 1C). Consequently, 30°C was selected as the optimal temperature to grow simultaneously *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV in a co-culture.

Synergistic properties of the co-culture of *Lactobacillus delbrueckii ssp. lactis* ATCC 15808 and *Geotrichum candidum VV* in various milk bases

Three milk bases -UHT milk-0/H (zero fat and hydrolyzed lactose) with or without autoclaving at 121°C for 10 minutes, and autoclaved (121°C for 10 minutes) reconstituted skim milk- were used for the investigations of possible synergistic interactions between *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV strains (Table 1). Low and high inoculation levels of VV compared to the levels of ATCC 15808 (t.f.u./ml:c.f.u./ml -ratios of 1:100 and 1:10, respectively) were tested; the growth of each culture at 30°C was followed by pH-measurements (Figure 2) and by specific platings for each component of the co-culture (Figure 3). A yoghurt starter like co-culture, consisting of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *Str. thermophilus* T101 strains (for which the initial inoculation ratio of T101:ATCC 15808 was about 1:20), as well as the single strain cultures were used as references.

As shown in Figure 2, the acid production (drop of pH-value) in the co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV with the high initial inoculation ratio of VV:ATCC 15808 (1:10) was comparable to the yoghurt starter like reference co-culture, *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *S. thermophilus* T101, at any time points, and in all three milk bases. This was also the case with the low initial inoculation ratio of VV:ATCC 15808 (1:100) for two autoclaved milk bases (Figure 2A and B). But, in the non-autoclaved UHT milk, the lower initial inoculum level of *G. candidum* VV retarded acid production compared to the higher inoculum of *G. candidum* VV (Figure 2C). Interestingly, just in this milk base,

Milk base	Producer	Fat (%)	Lactose[3]	Heat treatment
UHT milk[1]-1.5/NH	CSA, Synolilait Lyon (France)	1.5	NH	UHT
UHT milk - 0/H	Valio Ltd. (Finland)	0	H	UHT
UHT milk - 1.5/H	Valio Ltd. (Finland)	1.5	H	UHT
Skim milk[2]	Sigma-Aldrich	0	NH	Autoclaved at 121°C for 10 min

[1]UHT: Ultra high temperature (more than 135°C for few seconds)
[2]Skim milk: 10% (w/v) reconstituted skim milk
[3]lactose non-hydrolyzed (NH) and hydrolyzed (H)

Table 1: Features of the milk bases used in this study.

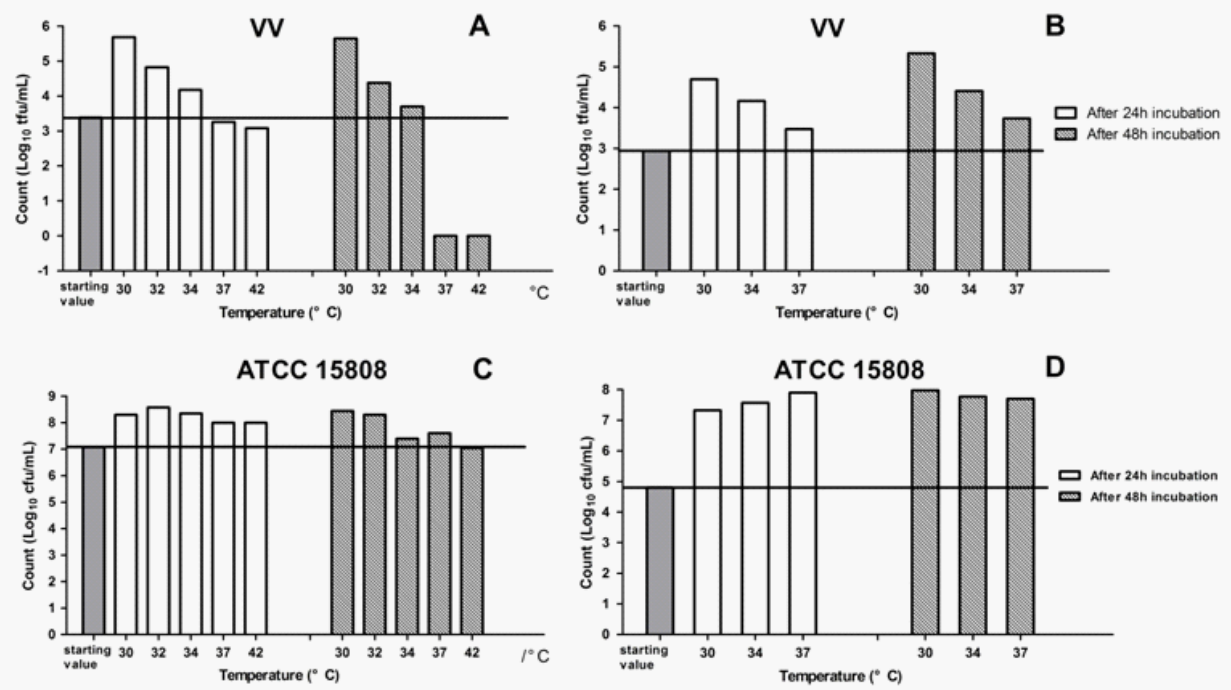

Figure 1: Determination of an optimal growth temperature for *G. candidum* VV (A and B) and *Lb. delbrueckii* ssp. *lactis* ATCC 15808 (C and D) grown in MRS broth. The enumeration of the cells was performed on MRS (pH 5.4) plates for strain ATCC 15808 and on YGC plates for strain VV at 37°C and 30°C, respectively, after 24 h and 48 h incubations. The horizontal line refers to the initial inoculation level applied (A and C: high level, B and D: low level).

the presence of *G. candidum* VV, at either low or high inoculation levels, stimulated the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 as detected from the pH values of the cultures. Noteworthy, the trend of the drop of the pH values over time, reflecting the synergistic growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 together with either *G. candidum* VV (high inoculum) (Figure 2C), or with *Str. thermophilus* T101 (Figure 2F) are fairly similar suggesting a comparable synergistic capability. Among the three considered milk bases, differences at c.f.u./ml or t.f.u./ml levels over time were most noticeable for either *Lb. delbrueckii* ssp. *lactis* ATCC 15808, or for *G. candidum* VV, when the growth studies were performed in non-autoclaved UHT milk (Figure 3). The growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 was stimulated by the presence of *G. candidum* VV at either low or high inoculum; the growth of ATCC 15808 increased by about two log units in the co-cultures (conditions b and c in Figure 3A), whereas a rise of about one log unit was observed when ATCC 15808 was grown alone (condition a in Figure 3A). The stimulatory effect by *G. candidum* VV looked equivalent to the one promoted by *Str. thermophilus* T101 (condition d in Figure 3A). The same non-autoclaved UHT milk enabled the growth of *G. candidum* VV, at either low or high inoculum, by about one log unit in the presence of *Lb. delbrueckii* ssp. *lactis* strain ATCC 15808 (Figure 3B). No real differences in the growth of *Str. thermophilus* T101 were observed with the tested milk bases, in the presence or absence of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 (Figure 3C).

Formic acid, a Growth Factor of Lactobacillus *delbrueckii ssp. lactis* strain ATCC 15808

As noticeable in Figures 2 and 3, the synergistic effects observed in the co-cultures of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV strains, and of ATCC 15808 and *Str. thermophilus* T101 strains were most obvious when the milk base was not subjected to severe heat treatments like autoclaving at 121°C for 10 minutes. In other words, strong heat treatments of the milk base appeared to yield some compound(s), which promoted the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808. The most obvious candidate is formic acid: it is known that formic acid is one of the major growth promoting metabolite delivered by *Str. thermophilus*, and it is required for the biosynthesis of purines [12,14,15,21]. Moreover, formic acid is also known to be one of the thermal degradation products of lactose [2]. Hence, we investigated whether formic acid would be a growth factor for *Lb. delbrueckii* ssp. *lactis* ATCC 15808. Based on the literature [15] and on our preliminary tests with 0.7 to 14.7 mM Na-formate (a dissociated form of formic acid, which exists at the pH of milk) supplements (data not shown), 7.35 mM Na-formate was chosen as the final formate supplement concentration in UHT milk (a non-autoclaved milk base) and in skim milk (autoclaved at 121°C for 10 min) for our growth factor analysis, performed both at 30°C and 42°C.

As revealed through pH measurements, growth promoting effects by 7.35 mM formate on *Lb. delbrueckii* ssp. *lactis* ATCC 15808 were only observed in the case of the non-autoclaved UHT milk base, at both temperatures (Figure 4A and B). The addition of formate, to autoclaved skim milk, did not show any stimulatory effect on the growth of ATCC 15808 at both investigated temperatures (Figure 4C and D). Altogether these results suggest that the autoclaving heat treatment of skim milk had replaced the formate through suggested partial thermal lactose degradation. Consequently, the effects of the autoclave heat treatment (121°C for 10 min) of milk bases and a 7.35

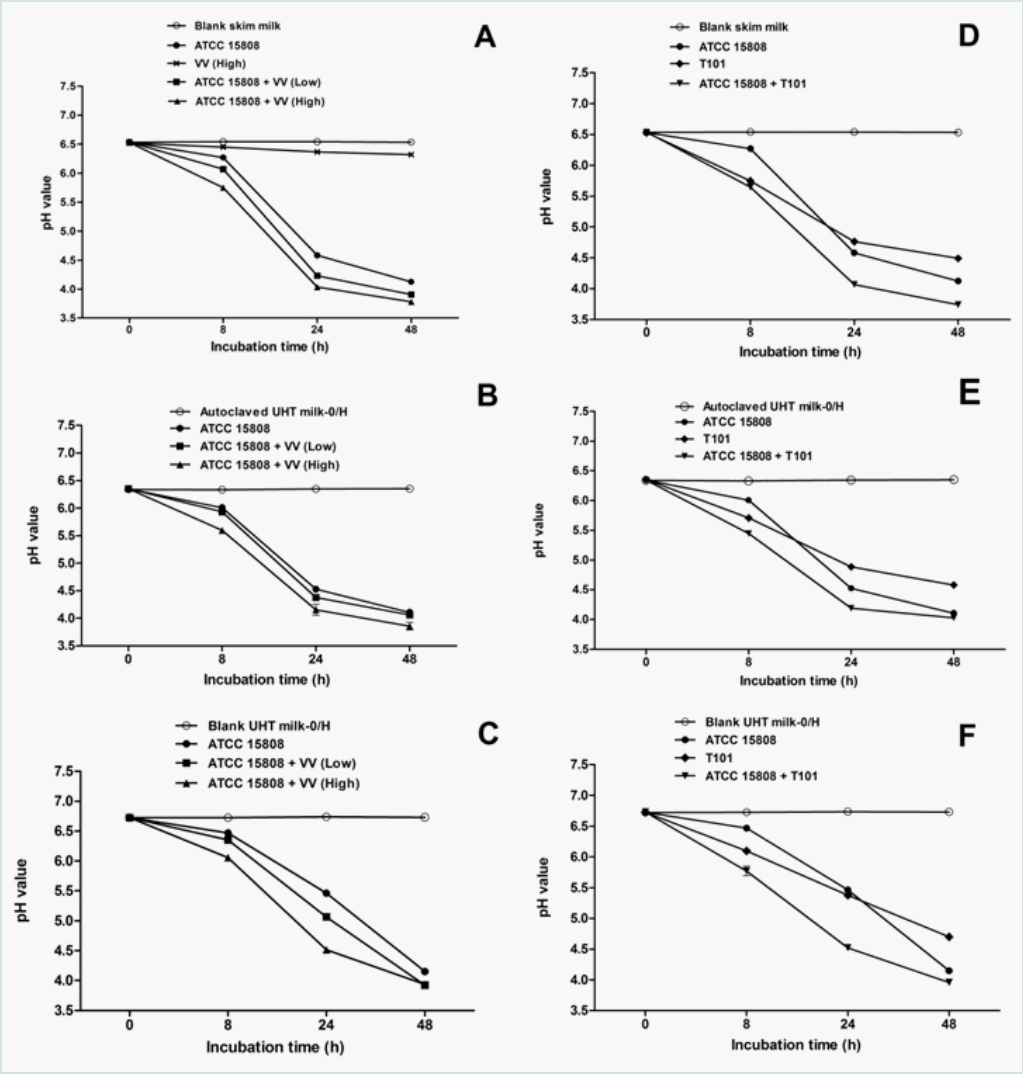

Figure 2: pH values of the co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV (A - C), and a yogurt starter like reference co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *S. thermophilus* T101 (D - F) as incubated at 30°C for 48 hours. Three milk bases as culture media were tested: Autoclaved (121°C/10 min) reconstituted skim milk (A and D), autoclaved (121°C/10 min) fatless, lactose hydrolyzed (0/H) UHT milk (B and E), and fatless, lactose hydrolyzed (0/H) UHT milk (C and F). Two inoculation levels of VV were included: 1) Low level: the ratio of VV:ATCC 15808 was about 1:100 2) High level: the ratio of VV:ATCC 15808 was about 1:10. The pH values were determined from three replicates.

mM formate supplement on the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 were compared in two UHT milk bases: UHT milk with non-hydrolyzed (NH) lactose, and UHT milk with hydrolyzed (H) lactose, having both similar fat content (1.5%). As shown in Figure 5, the autoclaving heat treatment caused similar growth promoting effects on *Lb. delbrueckii* ssp. *lactis* ATCC 15808, as did the 7.35 mM formate supplement for the UHT milks considered here. Altogether the growth promoting effect of the 7.35 mM formate supplement on *Lb. delbrueckii* ssp. *lactis* ATCC 15808 was independent on fat content, lactose hydrolysis or growth temperature.

Supplements of 5% CO_2 in air, 10-100 mM $NaHCO_3$ and 5-100 mM Na_2CO_3 in skim milk were tested as growth factors for *Lb. delbrueckii* ssp. *lactis* ATCC 15808. None of these additional compounds tested-carbon dioxide in gas phase or dissolved inorganic (bi) carbonate - did promote the growth of strain ATCC 15808 (data not shown).

Discussion

We investigated here whether there was a microbiological basis for the construction of a novel type of fermented milk, which would be a "hybrid" product between "Viili" and yogurt. A traditional "Viili" starter is based on the combination of mesophilic *Lactococcus* and *Leuconostoc* strains and *Geotrichum candidum,* a yeast-like white mold. LAB strains are responsible for lactic fermentation, citrate-based aroma formation and for characteristic high viscosity and ropiness of the "Viili" product. *G. candidum* mold creates a velvet-like creamy surface on "Viili". Typically, in the manufacture of "Viili", the fermentation takes place in the package and lasts about 18 to 20 h at 18-20°C [22]. On the other hand in the (set or stirred) yoghurt manufacture, the fermentation by a yogurt starter, which contains thermophilic *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* strains, typically lasts 2 to 4 h at 42-44°C,

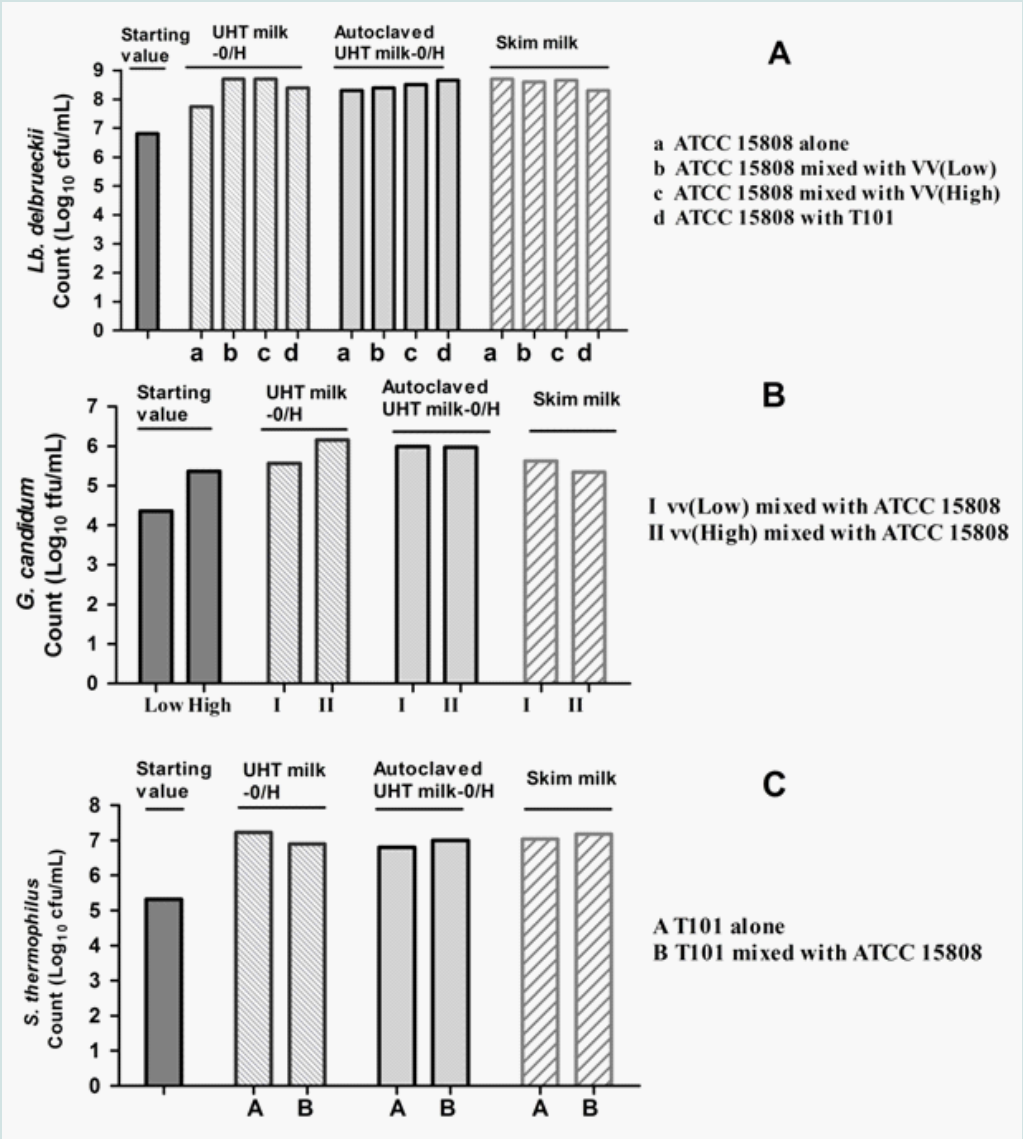

Figure 3: Growth of the co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV (A and B), and a yogurt starter like reference co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *Str. thermophilcus* T101 as incubated at 30°C for 24 hours. Milk bases and inoculation levels of VV were as described in Figure 2. Platings for counting *Lb. delbrueckii, Str. thermophilus* and *G. candidum* were done on MRS (pH 5.4), M17 and YGC agars as described in Materials and Methods.

or longer times at lower temperatures [2,23]. Yogurt starter species, the growth of which is known to show mutual stimulation, are responsible for lactic fermentation, and productions of yogurt aroma (especially acetaldehyde) and possible exopolysaccharides (EPSs) [2].

The replacement of the mesophilic LAB strains in a typical "Viili" starter by a single thermophilic *Lb. delbrueckii* strain, and the subsequent combination of this single strain with *G. candidum* was possible as the strains ATCC 15808 and VV shared a temperature range of 30-34°C (Figure 1), which allowed the simultaneous growth of both strains; we selected 30°C as the optimal temperature. This choice is consistent with the literature data, which reports that 100% of the *G. candidum* isolates were able to grow at 30°C, but only 1% of the isolates grew at 37°C [24].

From the results in Figures 2 and 3, the co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *G. candidum* VV showed similar synergistic properties as the yogurt starter like reference co-culture of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 and *Str. thermophilus* T101 when growing at 30°C in various milk bases. Formic acid (present as formate at the pH of the milk) and CO_2 produced by *Str. thermophilus* have been described to be the major compounds, which promote the growth of the second yogurt starter species, *Lb. delbrueckii* ssp. *bulgaricus* [2,25]. We demonstrated that formate, supplemented at 7.35 mM, could substitute the growth promoting effect of *Str. thermophilus* T101 or of *G. candidum* VV on *Lb. delbrueckii* ssp. *lactis* ATCC 15808 in UHT milk, but not anymore in skim milk, which had been autoclaved at 121°C for 10 minutes (Figure 4). As shown in Figure 5, a severe heat treatment like the autoclaving at 121°C for 10 minutes of the milk base was able to replace the formate supplement, obviously due to formic acid formation, through partial thermal

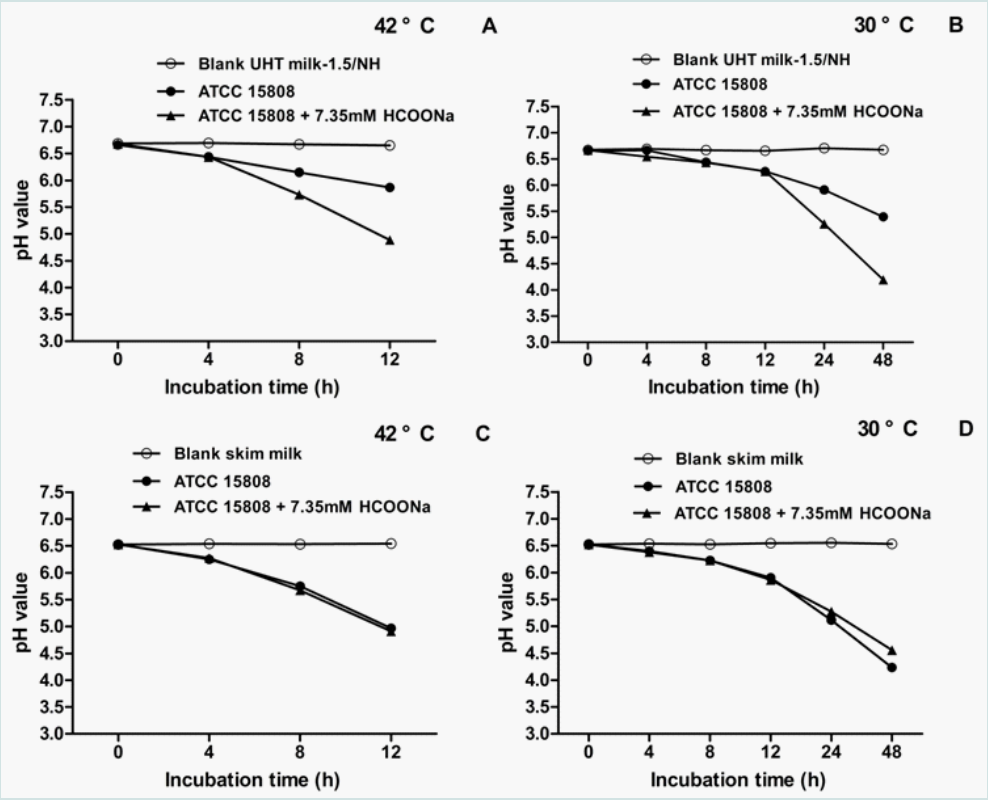

Figure 4: Effects of the 7.35 mM sodium formate (HCOONa) supplement in UHT milk (non-autoclaved) (A and B) and skim milk (autoclaved at 121°C for 10 min) (C and D) on the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 incubated at 42°C (A and C) and 30°C (B and D) as based on pH-values of the cultures. UHT milk-1.5/NH: lactose non-hydrolyzed UHT milk containing 1.5% fat; skim milk: reconstituted (10% w/v) skim milk (autoclaved at 121°C for 10 minutes).

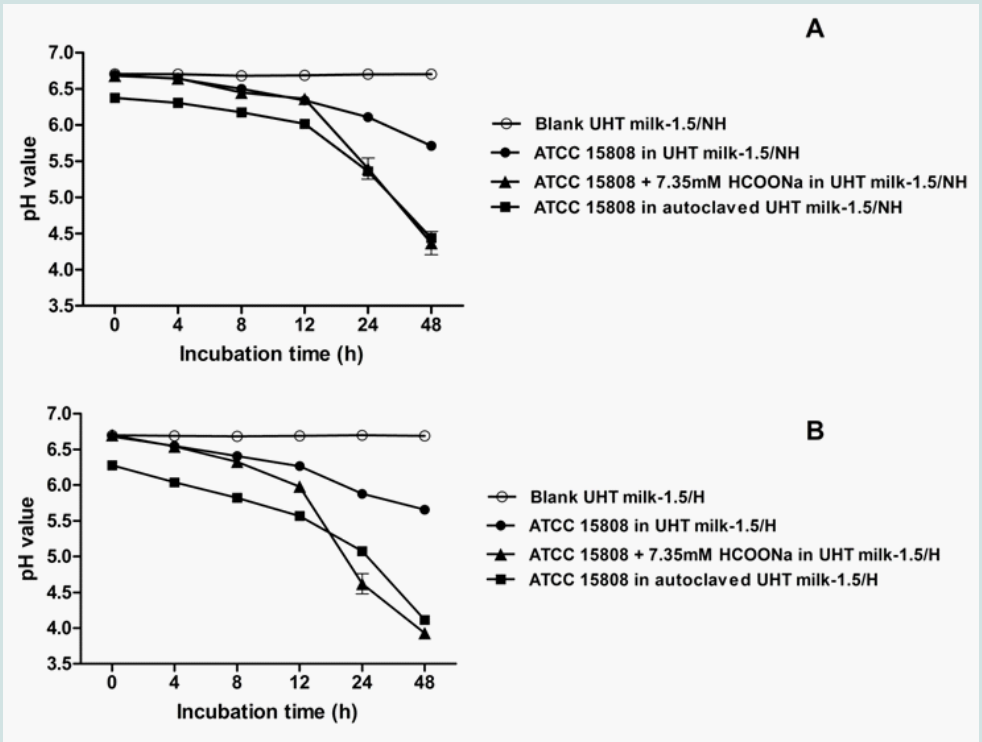

Figure 5: Effects of autoclave heat treatment (121°C for 10 minutes) of lactose hydrolyzed and lactose non-hydrolyzed UHT milks containing 1.5% fat (UHT milk-1.5/H and UHT milk-1.5/NH, respectively) on the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 incubated at 30°C as based on pH-values of the cultures.

lactose degradation [2]. Based on our observations, we suggest that *G. candidum* is able to replace successfully *Str. thermophilus* for the metabolic formic acid production required for the growth of *Lb. delbrueckii*. We could not observe any stimulatory effects on the growth of *Lb. delbrueckii* ssp. *lactis* ATCC 15808 by (bi) carbonate or CO_2 supplements (data not shown), even though the respirative metabolism by *G. candidum,* required for its growth, generates CO_2 at the cost of dissolved oxygen (O_2). *G. candidum* cannot metabolize lactose; but lactose fermentation by *Lb. delbrueckii* will produce a suitable carbon source, lactic acid, for the growth of *G. candidum* in the co-culture in a milk base. In addition to formic acid, *G. candidum* may synthesize other compounds, like unsaturated fatty acids, which are essential for the growth of *Lb. delbrueckii* [26]. Furthermore *G. candidum* may create an anaerobic environment for *Lb. delbrueckii* by consuming dissolved oxygen in the milk base, and by that way improving the growing conditions of the bacterial partner.

In this study, we have shown that a "Termoviili" starter like co-culture consisting of *Lb. delbrueckii* ssp. *lactis* and *G. candidum* strains is exhibiting similar synergistic properties as a yogurt starter like co-culture when growing in a milk base, where no or very low thermal lactose degradation occurred. The manufacture of "Termoviili" would take about the same time as the manufacture of the traditional "Viili", but the incubation temperature should be about 10-12°C higher, around 30°C. Contrarily to the standard yogurt manufacture, the milk for "Termoviili" should not be homogenized, and the fermentation should happen in the package in order to produce the velvet like cream layer covered with *G. candidum* in the final product like in the case of "Viili". Of crucial importance for the success of a novel type of fermented milk are its sensory and nutritional properties. Our very preliminary data on sensory properties of "Termoviili" indicated a flavor with slightly sharp, light alcohol, yeast/musty and organic acid flavor features, judged different from a typical yogurt flavor, but acceptable by all five panel members (data not shown).

In principle, the citrate catabolism based diacetyl flavor production by mesophilic LAB in "Viili" is replaced by yogurt aroma (mainly acetaldehyde) production by *Lb. delbrueckii* in "Termoviili". The slight sharpness of the flavor may be due to CO_2 production or consequent to lipolysis by *G. candidum*. The characteristic high viscosity property of "Viili" is based on the presence of ropy LAB (especially *L. lactis* ssp. *cremoris*) starter strains responsible for EPS (slime) production. Hence by applying EPS producing *Lb. delbrueckii* starter strain(s) and, if required, additional adjunct culture(s), the desired texture and flavour properties of "Termoviili" could be modified and optimized. As compared to yogurt, the presence of *G. candidum* could improve the nutritional properties of "Termoviili" through the consumption of dissolved O_2 (anti-oxidative environment) and through the synthesis of some vitamins and other valuable bioactive compounds by *G. candidum.*

At first, the manufacture of "Termoviili" would be as simple as "Viili"; but because there is no need to homogenize the milk fat, and because there is no need to control the ratio of the two thermophilic LAB starter species during milk fermentation due to the elimination of *Str. thermophilus*, one could consider milk less processed, and the manufacture of "Termoviili" hence looks even simpler than the manufacture of yogurt, even for a set type yogurt. Further studies with additional starter strains of both *Lb. delbrueckii* and *G. candidum* are needed to develop optimal starters, and finally a novel fermented milk product, "Termoviili".

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