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Yoghurt-Like Drink Enriched with Lactobacilli and Bifidobacteria †

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Abstract: To develop an enriched fermented milk an experimental design was used in a first step, by combining *Lactiplantibacillus plantarum* (isolated from oenological matrix) and *Bifidobacterium animalis* subsp. *lactis* strains, at different inoculum levels (4, 5 and 6 log CFU/mL) and temperatures (30, 35 and 40 °C) to study the effects on the acidification. Then, for the realization of the yoghurt-like drink, 5 log CFU/mL of both strains were inoculated in milk + 10% honey at 35 °C for 24 h; the fermented milk was stored at 4 °C for 50 days. The microbial concentrations were always > 8 log CFU/mL.

Keywords: probiotics; fermented milk; experimental design

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1. Introduction

The creation of a product requires a careful and scrupulous study of all the variables and an evaluation of how these can influence the final product. In literature many papers focus on the production of drinks added with probiotic or prebiotic [1–3]. This type of products responds to the constant demand of consumers who are increasingly attentive to the nutritional and health characteristics of foods. Yoghurt is one of the oldest foods and today it has become a trendy product, in terms of nourishment and versatility, considered a healthy protein food and a valid alternative to milk [4]. Like other fermented drinks, yoghurt brings several benefits to our body; it has anti-inflammatory and purifying properties, the acidity of the yoghurt favors the growth of an intestinal bacterial biota capable of counteracting the putrefactive phenomena that occur in the intestine. Many functions are performed by the synergistic action of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [4], however, different bacteria (e.g., probiotics) can be added.

The aim of this work was the formulation of a yoghurt-like drink with Lactobacilli and Bifidobacteria, through the use of microorganisms isolated from oenological matrices of the Apulian territory; an experimental design, the Centroid, was used to optimize the product.

2. Materials and Methods

Microorganisms and Milk: *Bifidobacterium animalis* subsp. *lactis* DSM 10140 (Deutsche Sammlung von Mikroorganismem und Zellkulturen's collection, Braunschweig, Germany) and one strain of *Lactiplantibacillus plantarum* coded as 33 isolated from Apulian (Italy) oenological matrix, were used in this research. The strains were stored at −20 °C in MRS broth (Oxoid, Milan, Italy) and MRS Broth + 0.5% cysteine, for *Lactiplantibacillus* and *Bifidobacterium*, respectively, with 33% (*V*/*V*) of sterile glycerol (J.T. Baker, Milan, Italy). Before each assay, the bacteria were grown twice in the same medium at 30 (*Lpb. plantarum*)

and 37 °C (*B. animalis* subsp. *lactis*) for 24 h; then, the cultures were centrifuged at 4000× *g* for 10 min at 4 °C, the supernatant was discarded, and the pellet re-suspended in distilled water. Apulian fresh whole pasteurized homogenized cow's milk (3.2 g/L protein; 4.9 g/L carbohydrates; 3.6 g/L fats) purchased from a local dealer was used.

Experimental design-Centroid: The optimization of the production of the yoghurt-like drink was performed through a centroid design [5,6]. In this research, the independent variables were the inoculum level of *Lpb. plantarum* 33 (L) and of *B. animalis* subsp. *lactis* (B), and temperature (T) (Table 1).

Table 1. Combination, coded levels and values of Centroid.

COMBINATIONS	CODED LEVELS			VALUES		
	B *	L **	T ***	B	L	T
A	1	0	0	6	4	30
B	0	1	0	4	6	30
C	0	0	1	4	4	40
D	0.5	0.5	0	5	5	30
E	0.5	0	0.5	5	4	35
F	0	0.5	0.5	4	5	35

* B = *Bifidobacterium animalis* subsp. *lactis*, CFU/mL; ** L = *Lactiplantibacillus plantarum* 33, CFU/mL; *** T = temperature, °C.

According to the design, *Lpb. plantarum* and *B. animalis* subsp. *lactis* were inoculated to 4, 5 and 6 log CFU/mL in 100 mL of pasteurized milk supplemented with honey at 10% (*V*/*V*); then, the samples were incubated at 30, 35 or 40 °C for 7 days. For each combination, microbiological sampling was carried out and the acidification was periodically monitored through pH measuring by a pH-meter (Crison, Barcelona, Spain). Each analysis was performed in duplicate.

Modeling: Microbial growth data (log CFU/mL), were plotted on an Excel worksheet and processed as mean value ± standard deviation.

Concerning pH, data were modeled as acidification (ΔpH), i.e., pH decrease referred to the beginning of the experiment; then, it was used as a dependent variable for a primary modeling through the "lag-exponential model" of van Gerwen and Zwietering [7] and Baty and Delignette-Muller [8] to obtain α, the time before the beginning of the acidification kinetic (h); d_{max}, the maximal acidification rate (1/h) and ΔpH_{max}, the maximum level of acidification.

Successively, ΔpH_{max} e d_{max} were used as input values for a multiple regression approach; temperature and inoculum levels of both strains were used as independent variables. The analysis was done through the software Statistica for Windows (StatSoft, Tulsa, OK, United States). The model was built by using the option "quadratic," for the evaluation of the individual ("*B. animalis* subsp. *lactis* inoculum level", "*Lpb. plantarum* 33 inoculum level" and "temperature") and interactive effects ("*B. animalis* subsp. *lactis* inoculum level * *Lpb. plantarum* 33 inoculum level; "*B. animalis* subsp. *lactis* inoculum level * temperature,"; and "temperature ∗ *Lpb. plantarum* 33 inoculum level"). Finally, the effect of each independent variable on the fitting parameters of the acidification kinetic (ΔpH_{max} and d_{max}) was evaluated through the individual desirability functions, as reported by Speranza et al. [3].

Yoghurt-like realization: 5 log CFU/mL of *Lpb. plantarum* 33 and *B. animalis* subsp. *lactis* were inoculated in 10 mL sample composed of 9 mL of milk and 1 mL honey and incubated at 35 °C for 24 h; then the samples were stored at 4 °C. Microbiological analyses and pH measurements were periodically assessed. For microbiological analyses the following media were used: MRS Agar acidified to pH 5 for *Lpb. plantarum* 33, and MRS Agar added with a solution of 4 antibiotics (Paromomycin sulphate 0.01 g, Neomycin

sulphate 0.005 g, Lithium 0.15 g, Nalidixic acid 0.75 g) for *B. animalis* subsp. *lactis*. The experiments were repeated twice on two independent samples [9,10].

3. Results

The microbial strains used, *B. animalis* subsp. *lactis* DSM 10140 and *Lpb. plantarum* 33 were preliminary analysed to evaluate their acidifying capability and subjected to adaptation trials to improve their performances [11]; then were inoculated in milk + honey at 10% (data not yet published). Successively, the product optimization was realised through an experimental design (Centroid).

3.1. Centroid

Table 2 shows the combinations of the experimental design and the performances of both strains referred to ΔpH_{max}, d_{max} and α. ΔpH_{max} varied from a minimum value of 2.20 (combination C) to a maximum of 2.59 (combination E); the acidification rate (d_{max}) ranged from 0.08 to 0.19 g^{-1}. The parameter α was statistically significant only for combinations A, B, C and D, ranging from 3.53 ± 0.61 (combination A) to 6.59 ± 1.15 days. The high standard error for B, C and D combinations suggests that acidification could occur even more rapidly.

Table 2. Acidification of *Lpb. plantarum* 33 and *B. animalis* subsp. *lactis*: fitting parameters of the "lag-exponential equation" (mean ± standard error).

COMBINATIONS				
	ΔpH_{max}	**d_{max}**	**α**	**R**
A	2.38 ± 0.01	0.08 ± 0.00	3.53 ± 0.61	0.999
B	2.24 ± 0.05	0.09 ± 0.00	5.19 ± 2.10	0.994
C	2.20 ± 0.07	0.19 ± 0.02	5.34 ± 2.00	0.980
D	2.34 ± 0.03	0.10 ± 0.00	6.59 ± 1.15	0.996
E	2.59 ± 0.06	0.11 ± 0.01	-	0.995
F	2.52 ± 0.07	0.11 ± 0.00	-	0.994

ΔpH_{max} = maximum acidification (decrease of pH at the end of the assay) d_{max} = maximal acidification rate (ΔpH/day); α = the time before the beginning of the acidification kinetic (d); R = regression coefficient.

Successively, ΔpH_{max} and d_{max} were used as dependent variables for a multiple regression approach. The first output was the table of the standardized effects, showing the statistical weight and the significance of each individual (inoculum of both microorganisms and temperature) and interactive factors.

Table 3 shows the standardized effects of the three variables on ΔpH_{max}. As individual term, *B. animalis* showed the greatest effect (73.814), followed by *Lpb. plantarum* 33 (69.330) and temperature (68.251). While the interaction "*Lpb. plantarum*-temperature" was the must significant, followed by "*B. animalis* subsp. *lactis*-temperature". Concerning d_{max}, the maximum effect was attributable to the temperature followed by the level of inoculum of *Lpb. plantarum* and *B. animalis* strains. The "*Lpb. plantarum*–temperature" and "*B. animalis* subsp. *lactis* –temperature" interactions were also significant.

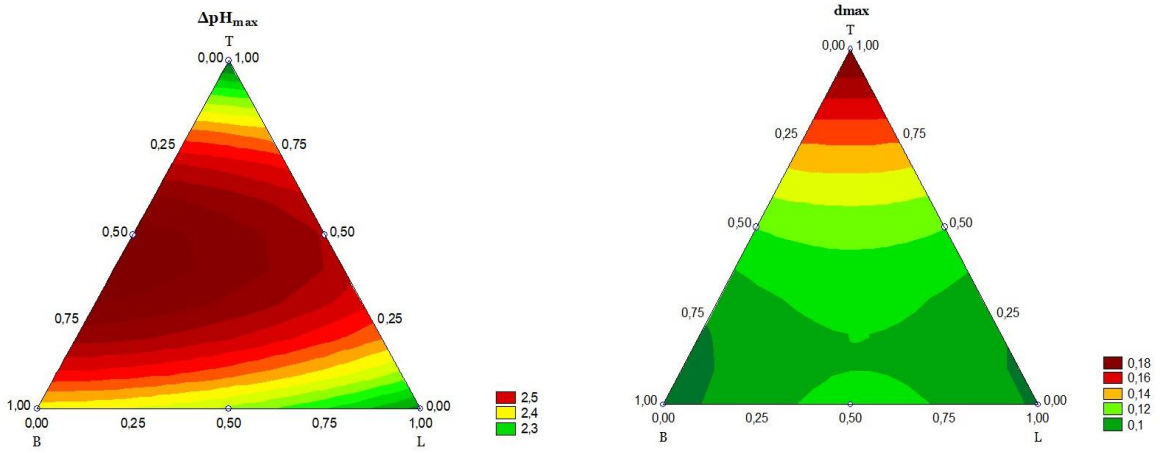
Table 3. Standardized effects of the inoculum of *B. animalis* subsp. *lactis* DSM 10140 [B], *Lpb. plantarum* [L] and temperature [T] on the maximum acidification (ΔpH_{max}) and on the acidification rate (d_{max}).

EFFECTS	**ΔpH_{max}**	**d_{max}**
[B]	73.814	12.746
[L]	69.330	13.429
[T]	68.251	28.331

[B] * [L]	- [A]	-
[B] * [T]	7.385	−2.999
[L] * [T]	7.614	−3.275
R^{2} [B]	0.901	0.930

[A] Not significant; [B] determination coefficient adjusted for the multiple regression.

Other outputs of centroid are the ternary plots; Figure 1a,b show the triangular surface for the effects of *B. animalis* subsp. *lactis* (B), *Lpb. plantarum* (L) and temperature (T) on the maximum acidification (ΔpH_{max}) (Figure 1a) and on the acidification rate (d_{max}) (Figure 1b). The model predicted the maximum acidification (ΔpH_{max} = 2.5) when either *Lpb. plantarum* or *B. animalis* and temperature were at the coded levels 0.5 (that is the microorganisms at 5 log CFU/mL and temperature at 35 °C) (Figure 1a). Concerning d_{max} (Figure 1b) the model predicted the highest values (d_{max} = 0.18) at the coded level 1 of the temperature (40 °C).



(**a**) (**b**)

Figure 1. Triangular graph relating to the effects of *B. animalis* subsp. *lactis* (B), *Lpb. plantarum* (L) and temperature (T) on the ΔpH_{max} (**a**) and d_{max} (**b**).

Triangular surfaces provide a quantitative assessment of interactive effects, but not of each individual term. This type of evaluation is possible through the desirability profile.

The desirability profile of ΔpH_{max} (Figure 2a) shows that both for *B. animalis* subsp. *lactis* and *Lpb. plantarum*, the increase of the inoculum level negatively affected acidification with a decreased performance of the strains at coded levels 0.75 or 1 (corresponding to 5.50 and 6 log CFU/mL). Concerning temperature, ΔpH_{max} was minimal at 30 °C and also decreased at coded levels 0.75 and 1 (corresponding to 37.5 and 40 °C).

The desirability profile of the acidification rate (d_{max}) (Figure 2b) shows that both *B. animalis* subsp. *lactis* and *Lpb. plantarum* 33 reduced the acidification rate at the inoculum coded level 1 (corresponding to 6 log CFU/mL). With regard to temperature, a positive correlation was observed: as temperature increased (for values coded as 0.75 and 1 = 37.5 and 40 °C, respectively), d_{max} increased.

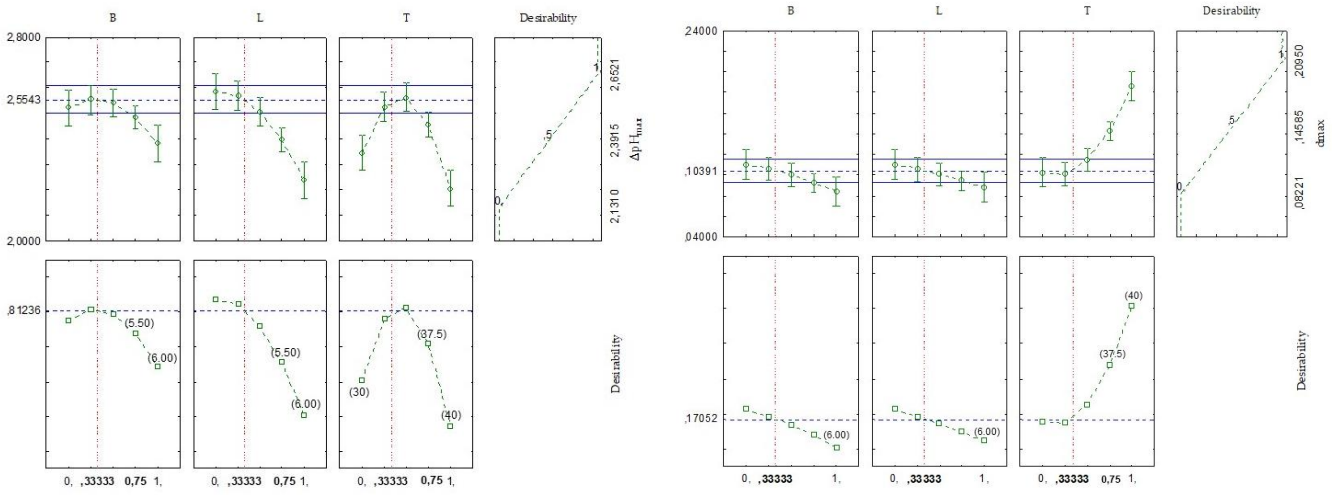


Figure 2. Prediction (upper side) and desirability profiles (down side) for the effect of inoculum level (log cfu/mL) of *B. animalis* subsp. *lactis* (B), *Lpb. plantarum* (L) and temperature as °C (T) on ΔpH_{max} (**a**) and d_{max} (**b**).

As shown by the desirability profiles (Figure 2a,b), the temperature acted differently on the maximum acidification and on the rate: in relation to the ΔpH_{max}, the increase in temperature led to a reduction in acidification, instead in the case of d_{max}, the increase in temperature was positively correlated with acidification rate. This phenomenon known as parameters uncoupling [12] highlights that increasing the temperature increases the acidification rate; however temperature cannot reach the maximum values because a reduction in acidification capability could occur.

3.2. Yoghurt-Like Drink

The main result of optimization of Centroid design was the best combination for obtaining the final product; as result of modelling the conditions were pointed out by software as follows: 5 log CFU/mL of *Lpb. plantarum* 33 and of *B. animalis* subsp. *lactis* 10,140 and temperature at 35 °C for 24 h. The concentration of both strains reached > 8 log CFU/mL. Then the samples were stored at 4 °C for 50 days and both strains were able to survive and never resulted below the criticality threshold (7 log CFU/mL) (Table 4). Furthermore, the pH did not undergo significant changes during storage (initial pH = 4.84; final pH = 4.22). Finally, the texture, odor and color of the yoghurt-like drink were optimal (data not shown).

Table 4. Cellular concentration (log CFU/mL) *Lpb. plantarum* 33 and *B. animalis* subsp. *lactis* DSM 10140 inoculated into the yoghurt-like drink and stored at a temperature of 4 °C. Lowercase letters indicate statistically significant differences along the column (effect of time for each strain); capital letters refer to the differences between the 2 strains at the same time of analysis (one-way ANOVA and Tukey's test, $p < 0.05$) Data are expressed as mean values ± standard deviation.

Time (Days)	33	10,140
0	8.78 ± 0.00 ^{a,A}	8.40 ± 0.00 ^{a,B}
1	8.74 ± 0.01 ^{a,A}	8.90 ± 0.02 ^{b,A}
4	8.90 ± 0.03 ^{a,A}	8.85 ± 0.00 ^{b,A}
6	8.93 ± 0.02 ^{a,A}	8.72 ± 0.03 ^{b,A}
19	8.88 ± 0.03 ^{a,A}	8.78 ± 0.02 ^{b,A}
26	8.85 ± 0.00 ^{a,A}	8.88 ± 0.03 ^{b,A}
33	8.52 ± 0.02 ^{b,A}	8.60 ± 0.00 ^{a,A}
40	8.74 ± 0.01 ^{a,A}	8.88 ± 0.02 ^{b,A}

50	8.74 ± 0.04 ^{a,A}	8.88 ± 0.03 ^{b,A}

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