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Using a simplex centroid to study the effects of pH, temperature and lactulose on the viability of *Bifidobacterium animalis* subsp. *lactis* in a model system

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ABSTRACT

This paper reports on the effects of lactulose (0–10 g/l) on *Bifidobacterium animalis* subsp. *lactis*, along with the influence of pH (4.5–8.5) and temperature (15–45 °C); the three factors were combined through a simplex centroid. The experiments were performed in a laboratory medium and the data of cells counts were modeled through the Weibull equation for the evaluation of the first reduction time, the shape parameter and the death time. These fitting parameters were used as input values to build a desirability profile and a second-order model through the DoE approach (Design of Experiments). The medium containing glucose was used as control.

The prebiotic enhanced the viability of the microbial target, by prolonging the first reduction time and inducing a shoulder phase in the death kinetic; moreover, in some combinations the statistical analysis highlighted a kind of interaction with the pH.

1. Introduction

A prebiotic was defined by Roberfroid as a non-digestible dietary component that undergoes selective colonic fermentation, “thus causing significant changes in the composition of the gut microflora with increased and reduced numbers of potentially health-promoting bacteria and potentially harmful species respectively” [1].

The prebiotics include several oligosaccharides (fructo-, gluco-, galacto-, isomalto-, xylo-, and soyo-oligosaccharides), inulin, lactulose, lactosucrose [2]. Lactulose is a synthetic disaccharide produced from the isomerization of the lactose [3]; it is normally used in the pharmaceutical industry, as an effective drug for the acute and chronic constipation [4,5]. Moreover, some promising applications were also reported in the nutraceuticals and food industries, due to the beneficial effects of lactulose on human health [6] or to its ability to stimulate the growth of probiotics [7–9]. De Souza-Oliveira et al. [5] reported a beneficial effect of lactulose on some bifidobacteria both in the growth and death phases; in addition, Saarela et al. [10] observed that lactulose improved the cold-storage stability of *Lactobacillus salivarius* at 4 °C for 22 days, probably due to the splitting of cell-chains. Other authors reported also a direct effect of lactulose on the β-glucosidase and β-galactosidase activities of the intestinal microbiota [11,12]. Lactulose was also proposed as an ingredient for synbiotic products [13]; however, in a food a prebiotic could interact with some parameters (pH, storage temperature, amounts of other nutrients, thermal treatments, etc.), thus its activity could be significantly affected, as well as its effect on the probiotic population.

To the best of our knowledge, nobody focused on this issue in the past; therefore, this paper was aimed to study the combined effects of lactulose with either pH or storage temperature through a mixture design. *Bifidobacterium animalis* subsp. *lactis*, strain DSM 10140, was chosen as the target microorganism and selected for the results of some preliminary experiments, as lactulose had showed a promising effect through the increase of the Growth Index and the decrease of the viability loss (Altieri et al., unpublished results).

2. Materials and methods

2.1. Strain

B. animalis subsp. *lactis* DSM 10140 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used throughout this research. The strain was stored at −20 °C in MRS broth (Oxoid, Milan) +0.05% of cysteine (Sigma–Aldrich, Milan) (cMRS), added with 33% of sterile glycerol; before each assay the strain was grown in cMRS broth incubated at 37 °C for 24 h.

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Table 1
Composition of the medium.

Lactulose/glucose	[a]
Yeast extract (Oxoid)	2.00 g/l
K_2HPO_4 (J.T. Baker, Milan)	2.70 g/l
$MnSO_4$ (J.T. Baker)	0.05 g/l
$MgSO_4 \cdot 7H_2O$ (J.T. Baker)	0.20 g/l
Tween 80® (C. Erba, Milan)	1.0 ml/l
Cysteine	0.5 g/l
pH	[a]

[a] Variable (see Table 2).

2.2. Medium and planning of the research

The experiments were performed in the laboratory medium proposed by Jyoti et al. [14] and Liew et al. [15], modified as reported in Table 1. The medium was added with either glucose or lactulose and inoculated up to 8 log cfu/ml with *B. animalis* subsp. *lactis*; the concentration of glucose or lactulose (0–10 g/l), and the pH of the medium (4.5–8.5) varied according to a simplex centroid, as reported in Table 2. The samples were stored at 15, 30, and 45 °C (Table 2) for 32 days under aerobiosis to mimic the normal storage of a beverage.

The viable count of *B. animalis* subsp. *lactis* was evaluated throughout the storage on cMRS agar, incubated at 37 °C for 72 h under anaerobic conditions; the pH of the samples was evaluated through a pH-meter Crison mod. 2001 (Crison Instruments Inc., Barcelona, Spain). The analyses were performed in duplicate immediately after the inoculation, after 24 and 48 h and then every other day; the experiments were performed in duplicate over two different batches.

2.3. Data modeling: first order model

The data of the viable count of *B. animalis* subsp. *lactis* were fitted through the Weibull equation, as modified by Mafart et al. [16], cast in the following form:

$$N = N_0 - \left(t/\delta\right)^p$$

where N_0 and N(log cfu/ml) are the initial cell count and the cell count throughout the time, respectively; δ, the first reduction time (days), i.e. the time to attain a reduction of 1 log cfu/ml in the cell count; p, the shape parameter. Data were fitted also through the Weibull equation, modified by Bevilacqua et al. [17] for the evaluation of the death time. The fitting parameters of the Weibull equation were analyzed through one-way analysis of variance (one-way ANOVA) and Tukey's test as *post-hoc* comparison test to highlight the significant differences.

2.4. Data modeling: second-order model and desirability function

The fitting parameters of the equation of Weibull, i.e. the parameters δ and p, were used as input values to build a second-order model through the option DoE of the software Statistica for Windows (Statsoft, Tulsa, Okhla.) to highlight the individual and interactive effects of pH, storage temperature, glucose/lactulose on the viability of *B. animalis*. Thereafter, the significance of the different terms of the simplex centroid was evaluated through the desirability function, as follows:

$$d = \begin{cases} 0, & y \le y_{\min} \\ (y - y_{\min})/(y_{\max} - y_{\min}), & y_{\min} \le y \le y_{\max} \\ 1, & y \ge y_{\max} \end{cases}$$

where $y_{\min}$ and $y_{\max}$ are the minimum and maximum values of the dependent variable, respectively.

The desirability is a dimensionless parameter, ranging from 0 (bad result) to 1 (good result in terms of cell viability).

Table 2
Combinations of glucose/lactulose, pH of the medium and storage temperature.

Runs	pH	T (°C)	Glucose/lactulose (g/l)
A	8.5	15	0.0
B	4.5	45	0.0
C	4.5	15	10.0
D	6.5	30	0.0
E	6.5	15	5.0
F	4.5	30	5.0

3. Results and discussion

B. animalis subsp. *lactis* was inoculated in a lab medium, containing either lactulose or glucose; the pH of the substrate was adjusted to different values and the samples stored at temperatures from 15 to 45 °C. Thus, two different designs were implemented based on the carbon source (glucose-G and lactulose-L); each design consisted of six different combinations. *B. animalis* subsp. *lactis* experienced an upward/downward trend, thus the data were fitted through the equation of Weibull; the fitting parameters are reported in Table 3.

The lack of a carbon source (combinations A_L and A_G) induced a prolonged viability of the target, with a first reduction time >32 days (running time); another interesting trait is the value of this parameter in the combinations C_L and E_L, containing respectively 10 and 5 g/l of lactulose. In fact, δ was 33 days, significantly higher than the values recovered in the same combinations with glucose (22.22 days in the run C_G and 18.62 days in the combination E_G).

The parameter p is a dimensionless parameter, connected with the shape of the curve; it is an important tool to obtain some information on the trend experienced by *B. animalis* subsp. *lactis*. A $p > 1$ stands for a downward curve, characterized by an initial phase with a slight or a not significant decrease in the cell count, similar to the shoulder length of the equation of Geereard et al. [18]; whilst a $p < 1$ indicates a tail effect, i.e. a fast decrease of the cell count in the first days, followed by a residual surviving

Table 3
Fitting parameters (±standard error) of the Weibull equation, by Mafart et al. [16], and death time [17] of *B. animalis* subsp. *lactis* in the different combinations of the design.

	δ[*]	p	Death time
Lactulose			
A_L	>32[**]	>5	>100
B_L	0.06 ± 0.09a[***]	0.36 ± 0.09a	21.89 ± 7.30b
C_L	32.79 ± 1.84d	3.96 ± 2.14c	86.08 ± 26.69d
D_L	3.52 ± 3.26a,b	0.70 ± 0.27a	72.98 ± 26.67d
E_L	33.02 ± 1.91d	3.67 ± 1.83b,c	84.01 ± 20.56d
F_L	9.92 ± 2.74b	1.71 ± 0.40a,b,c	34.14 ± 2.13c
Glucose			
A_G	>32	>5	>100
B_G	0.00 ± 0.00a	0.14 ± 0.01a	4.00 ± 0.11a
C_G	22.22 ± 1.03c	4.01 ± 0.50c	37.61 ± 0.93c
D_G	3.55 ± 4.17a,b	0.66 ± 0.32a	76.46 ± 15.94d
E_G	18.62 ± 4.15c	1.04 ± 0.33a,b	81.28 ± 17.79d
F_G	0.72 ± 0.89a	0.62 ± 0.19a	21.83 ± 5.80b

[*] δ, First reduction time (days); p, shape parameter.
[**] The value of the parameter was higher than the running time (32 days).
[***] For each parameter the letters indicate the significant differences (one-way ANOVA and Tukey's test, $P < 0.05$).