

Kinetic Parameters of Lactic Acid Growth and Production for *Bifidobacterium lactis* BB12 in Cabbage Juices

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The suitability of the cabbage juice for Bifidobacterium sp. growth was investigated. Cabbage juices fresh, heat-treated, respectively filtered and heat-treated were subject to lactic acid fermentation in the same conditions. Chemical and microbiological parameters were determined in dynamics: pH, lactic acid, acetic acid, reducing sugars, biomass and viable cells count, through methods standardized or quoted in literature. The maximum rate of acidification v_{max} ranged between 2.66 to 5.58, while the time to reach pH 5.0 (hours) and the maximum volumetric productivity were 8 and 13.1×10^{10} CFU/L·h respectively in the case of the heat-treated juice. A maximum rate of acidification, by $0.076923h^{-1}$, was obtained for the filtered cabbage juice. No very significant differences concerning the cell viability after 24h were registered, this one varying between 9.08 to $9.5 \log$ CFU/mL.

Keywords: probiotic juices, lactic acid fermentation, Bifidobacterium sp., vegetables, kinetic parameters

Cruciferous vegetables are among the most important dietary vegetables consumed in Central European countries owing to their availability at local markets, low cost and consumer preference [1]. Cabbage is a cruciferous vegetable, which is rich in minerals, vitamin C, dietary fibers, and especially photochemicals [2]. The cabbage, vegetable very used in Romanian cuisine also, can be used as natural antioxidant source due to the amounts of polyphenols, flavonoids and anthocyanins, with strong antioxidant activity [3].

The food industry is directing new product development towards the area of functional foods and functional food ingredients due to consumers' demand for healthier foods [4]. The rise of functional foods has occurred at the convergence of several critical factors, such as: awareness of personal health deterioration, led by busy lifestyles with poor choices of convenience foods and insufficient exercise; increased incidence of self-medication; increased level of information from health authorities and media on nutrition and the link between diet and health; scientific developments in nutrition research; and a crowded and competitive food market, characterized by pressurized margins [5].

A probiotic is defined classically as a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract [6]. For human nutrition, the following definition has been proposed: "a live microbial food ingredient that is beneficial to health" [7]. Bifidobacteria, naturally present in the dominant colonic microbiota, represent up to 25% of the cultivable faecal bacteria in adults and 80% in infants. As probiotic agents, bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, and colonic adenomas and cancer [8]. Specific strategies for modifying gut microbiota in favour of bifidobacteria could be useful

tools for reducing the impact of high-fat feeding on the occurrence of metabolic diseases [9].

Nondairy probiotic products have a big worldwide importance due to the ongoing trend of vegetarianism and to a high prevalence of lactose intolerance in many populations around the world. Lactose intolerance, cholesterol content, and allergenic milk proteins are the major drawbacks related to the intake of dairy products, which makes the development of new nondairy probiotic foods essential [10]. Bifidobacterium species and lactic acid bacteria, especially Lactobacillus strains, are widely used in food production, not only in fermentation of vegetables, sausages, and milk, but also in fruit-based and vegetable-based products, such as carrot, beet, and celery [11].

Application of probiotic cultures in nondairy products represents a great challenge. It is important that the formulation maintains the activity and viability of the probiotic for extended periods of time [12]. Factors like water activity, oxygen tension, and temperature become increasingly important when dealing with these kinds of products [10].

Bifidobacteria are fastidious bacteria that require complex and expensive media for propagation [13]. *Bifidobacterium animalis* subsp. *lactis* is one of the most usual industrial strains due to its industrial properties such as tolerance to oxygen and acid resistance [14].

Mathematical modelling is an important tool to optimize fermentation processes [13]. Considerable amount of research was directed towards answering the questions of how to parametrize the optimal control problem, how to choose the admissible values for control to generate the grid points, at what rate to reduce the region size, how to handle state constraints, how to ensure that the global optimum was obtained, etc. [15]. The unstructured models are easier to use, and have been proved to describe accurately lactic acid fermentation in a wide range of experimental conditions and media [16].

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Experimental part

Bifidobacterium animalis subsp. *lactis* BB-12 was obtained from Christian Hansen (Romania).

Fresh cabbage was purchased from a local store at the beginning of December and specifically processed by removing the non-edible pieces. Using a home-made extractor, the vegetables were transformed in juice.

The lyophilized culture was aseptically added in proportion of 0.2g/L to the cabbage juice and vigorous homogenized for 15 min. Three experimental batches were performed:

- UT - cabbage juice heated at 40 degrees C and inoculated with 0.2g/L pure culture

- TT - cabbage juice thermal treated at 80 degrees C, cooled at 40 degrees C and inoculated with 0.2g/L pure culture

- FT - cabbage juice filtered, thermal treated at 80 degrees C, cooled at 40 degrees C and inoculated with 0.2g/L pure culture

100mL juice from each experimental batch was distributed in sterile tubes. The anaerobiosis was created by covering the cotton stopper of the tube by metal folia. Each tube was represented a single sample and the experiments were performed in double.

The lactic acid fermentation was performed in a thermostat at $37 \pm 0.2^\circ\text{C}$. The samples were analyzed during the process dynamic through chemical and microbiological analysis.

The count of *Bifidobacterium* sp. was determined by plate count method using Man-Rogosa-Sharpe agar, enriched with L-cysteine HCl, after serial tenfold dilutions in peptone water. The Petri dishes were incubated for 48h at 37°C in anaerobiosis (Anaerocult® C- anaerobiosis generator from Merck). The results were expressed as CFU/ml juice.

The optical density of biomass was measured with the UV-Visible spectrophotometer at 610nm. In the preparation of the calibration curve for optical density vs. dry cell weight several dilutions of the samples were made. According [17], for each dilution 2 mL of sample was used to obtain optical densities at 610 nm wavelength and 15 mL of sample was filtered with a pre-weighed cellulose acetate membrane filter having a pore size of 0.45 μm using a vacuum pump. The biomass collected on the filters was washed with 15 mL of water and the filters were dried at 100°C for approximately 24 h until constant weight was observed.

The pH values were measured with a HACH pH-meter. From the chemical point of view, the titratable acidity, expressed in g lactic acid/100mL, was determined by titration with NaOH 0.1N in the presence of phenolphthalein, while the values of the volatile acidity, expressed in g acetic acid/100mL, were established by steam distillation. The reducing sugars were analyzed applying the spectrophotometric method with 3,5-dinitrosalicilic acid (DNS) after the sample defecation with basic lead acetate. The results were expressed in g glucose/100mL.

An unstructured unsegregated model was used. The kinetic model was based on three rate equations, as following: *biomass growth*, *substrate utilization* and *product formation*.

According [17], a lot of previous studies were included the model which describes the rate of increase in biomass (dX/dt) as a function of the biomass only:

$$\frac{dX}{dt} = \mu X$$

where X is the biomass concentration (g/L) and μ the specific growth rate (h^{-1}).

The specific growth rate μ was expressed as a function of the limiting substrate concentration by a Monod equation:

$$\mu = \frac{\mu_{\max} S}{K_s + S}$$

where μ_{\max} is the maximum specific growth rate (h^{-1}), S is the substrate concentration (g/L) and K_s is the saturation constant or the Monod constant (g/L).

A plot of $1/\mu$ versus $1/S$, which is also known as a Lineweaver-Burk plot, yields a linear line with a slope of K_s/μ_{\max} and y-axis intercept of $1/\mu_{\max}$.

The kinetics of the substrate utilization for the lactic acid fermentation of the cabbage juice with *Bifido-bacterium* sp. was underlined by the biomass and product yield coefficients on substrate (Y_{XS} , respectively Y_{PS}), defined as stoichiometric coefficients:

$$Y_{XS} = \frac{X_f - X_0}{S_0 - S_f}$$

$$Y_{PS} = \frac{P_f - P_0}{S_0 - S_f}$$

The specific growth and production rates were obtained from time-derivation of growth data and from production rates, respectively [16].

Concerning the product formation, lactic acid fermentation was described by [18]. According to this model the instantaneous rate of lactic acid formation (dP/dt) can be related to the instantaneous rate of bacterial growth (dN/dt), and to the bacterial density (N):

$$\frac{dP}{dt} = \alpha \frac{dN}{dt} + \beta N$$

where the constants α (the growth associated product formation) and β (the non growth associated product formation) are determined by the pH of the fermentation.

A simplified presentation of the above model relates to the linear part of the equation was presented by [19]:

$$(P - P_0) = a(X - X_0)$$

where P_0 and P are the concentrations of lactic acid (g/L) initially and at time t , respectively, and X_0 and X are the increases of the biomass (log CFU/mL) initially and at time t , respectively.

The experimental data were analyzed using SPSS Statistics 17.

Results and discussions

During 24h of lactic acid fermentation of cabbage juice with *Bifidobacterium* sp., the pH values were ranged between 6.7 and 4.15 (fig.1), while the titratable acidity was increased from 0.83g lactic acid/L to 5.3 g lactic acid/L (fig.2).

In the first 2 h of the process, the pH decreasing was significant in the case of the sample without thermal treatment (UT), probably due to the activity of the epiphytic microbiota. This value was 2 times and 3 times higher than in the case of the samples TT and FT respectively. Only after 6 h these differences become almost insignificant, because the bacteria had time to adapt to the new environment. After 24 h, the cabbage juice thermal treated has registered a pH decrease with 2.01 units, while this difference was by 1.73 units in the case of the untreated juice.

Sample	$\mu_{\max}(\text{h}^{-1})$	K_s (g/L)	α_{exp} (g/g)	α_{calc} (g/g)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)
UT	0.053191	0.02534	0.467	0.967	0.021306452	0.102419355
TT	0.0625	0.01773	1.22	0.904	0.01756044	0.241758242
FT	0.076923	0.02732	0.797	0.869	0.01404878	0.14

Table 1
KINETIC PARAMETERS VALUES

The maximum rate of acidification v_{\max} was calculated as the time variation of pH (dpH/dt) and expressed as pH units/min. The highest maximum rate of acidification was obtained in the case of the batch UT, followed with closed values by TT and FT (5.58, 2.75 and 2.66 respectively). The time to reach v_{\max} (t_{\max} , hours) was although quite different: 2 h in the case of the samples UT and TT, respectively 8 h in the case of the batch FT.

Other kinetic parameters were also calculated: time to reach pH 5.0 ($t_{pH\ 5.0}$, hours) and time to complete the fermentation ($t_{pH\ 4.2}$, hours). The time to reach pH 5.0, important parameter concerning the stability of the final products, was varied between 8 (TT) and 23.29 (UT). For the cabbage juice filtered and thermal treated (FT), the indicator had a relative good value, by 10.42. The best value for time to complete the fermentation ($t_{pH\ 4.2}$, hours) was obtained for the fermentation of the thermal treated cabbage juice (TT), this one being by 23.05. It is also important to specify that after 72 h in both samples UT and FT the pH has decreased until values close to 4.2. In the case of the cabbage juice without thermal treated it seems that bifidobacteria are more competitive in relation with the epiphytic microorganisms.

Figure 2 shows the evolution of the titratable acidity expressed as lactic acid. As it can be observed, the values were comparable for all the cabbage juice fermentations in the first 6 h. After this period of time a lot of differences become noticeable, the volatile acidity being important in the mentioned context. It is known that the fermentation of hexoses occurs in the genus *Bifidobacterium* through bifid shout [20]. *Bifidobacteria* are saccharolytic species and they produce acetic acid and lactic acid (3/2, without CO_2). Some researchers have proposed a theoretical molar ratio of acetic acid to lactic acid of 1.5, although other scientists have proven that this ratio is not always obtained [21, 22]. The increasing of the lactic acid during 24 h was not comparable for all fermentations, being by 124.5% for UT, 345.78% for FT and 488.88% for TT respectively.

After 8 h of fermentation a higher pH was correlated with a relative high amount of lactic acid (1.89g/L) in the case of the batch UT, while the sample TT was characterized through a lower pH and a quantity by 1.66 g lactic acid/L. The evolution of the volatile acidity can explain this proportionality, the quantity of the acetic acid being less than half in the thermal treated cabbage juice (1.2 g acetic acid/L for UT and 2.56 g acetic acid/L respectively for TT). The influence of the type of the organic acid resulted as metabolit on the pH values of juices is not so obvious due to the smaller difference between the pK_a values of acetic acid and lactic acid (about 0.05 unities). In the interval 8-24 h the changes were yet significant.

The initial content of the substratum was strong influenced by the treatment applied before the fermentation of the cabbage juice (fig.3). In this respect, the heating at 80 degrees C for 10 min was induced a decrease of the reducing sugars with 29.13%, while the filtration followed by thermal treatment caused losses by 20.43%.

The consumption of the reducing sugars after 24 h of lactic acid fermentation with *Bifidobacterium* sp. was

ranged between 26.95% for UT and 56.01% for FT. In the case of the batch TT this parameter was close to the last, being 55.82%. The slowly decreasing of the fermentescible sugars during this period of time was correlated with the lent increasing of the titratable acidity.

The kinetic model describing microorganism growth and product formation is based on the carbon source in its entirety, especially that the product variation is the important aspect for further optimal control of the process. [23].

The viable cell count for all the experimental batches versus fermentation time is represented in figure 4. After a short latency in the cases of the batches TT and FT, no significant differences concerning the cell viability after 24h were registered, this one varying between 9.08 to 9.5 log CFU/mL.

The maximum volumetric productivity was by 13.1×10^{10} CFU/L·h for the heat-treated juice. This parameter registered very close values in the case of the other batches: 5×10^{10} CFU/L·h for the cabbage juice filtered and thermal treated (FT) and 5.25×10^{10} CFU/L·h for the cabbage juice untreated thermal (UT).

The relationship between the specific growth rate and the substrate concentration was represented for each experimental sample (fig.5). The Lineweaver-Burk plot was used with a view to determine the values of the half-velocity constant K_s .

The kinetic parameters of the fermentation of the cabbage juice in all the batches, including the modelled growth associated product formation (α_{calc}) are summarized in table 1. The lowest value of the saturation constant was obtained in the case of the sample heat-treated ($K_s = 0.01773\text{g/L}$), while very small differences were established between the other two.

Applying a non-linear regression in the fermentation of glucose by *L. delbrueckii*, [24] was obtained a $K_s = 0.0967\text{g/L}$, while a higher value of the same parameter, by 1.32, was obtained by [25] in the growth of *L. lactis* NZ133 on lactose.

An average value of the maximum specific growth rate was obtained in the case of the cabbage juice thermal-treated (0.0625 h^{-1}). A close value, by 0.0696, was determined also for *L. delbrueckii* [24], while 0.25, respectively 0.82 were obtained for *L. helveticus* by [26] and [27].

The growth associated product formation ranged in large intervals, different values being obtained by different authors. For example, $\alpha = 13.2\text{ g/g}$ in the fermentative production of lactic acid from glucose by *Lactococcus lactis* ssp. *lactis* ATCC 19435 in whole-wheat flour [28], while in the growth of *Lactobacillus delbrueckii* on beet molasses [29] was determined a lower value, by 0.235.

The cell yield coefficients ($Y_{X/S}$) were relative comparable for all the fermentations, these values being until 10 times smaller than those determined by other authors, in different environmental conditions. $Y_{X/S}$ varied between 0.17 and 0.31 g of CDM g of substrate⁻¹ when the growth of *Bifidobacterium animalis* DN-173 010 on different energy sources was studied through small- and large-scale

		glucose	lactic_acid	pH	viable_cells	biomass
glucose	Pearson Correlation	1	-.737**	.847**	-.674**	-.661**
	Sig. (2-tailed)		.000	.000	.002	.003
	N	18	18	18	18	18

Table 2
PEARSON COEFFICIENTS FOR THE
EXPERIMENTAL BATCHES

** . Correlation is significant at the 0.01 level (2-tailed).

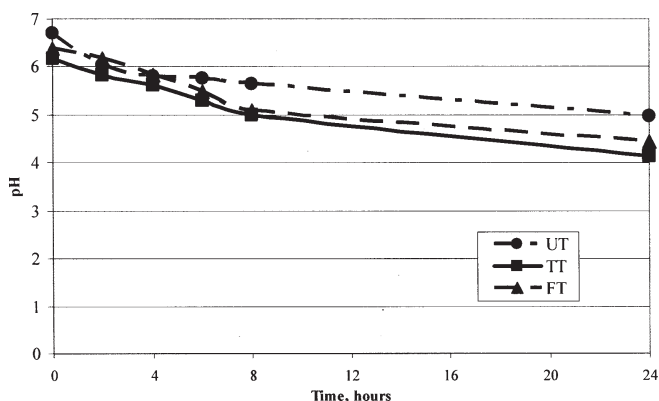


Fig. 1. pH dynamics during the lactic acid fermentation of the cabbage juice with *Bifidobacterium* sp

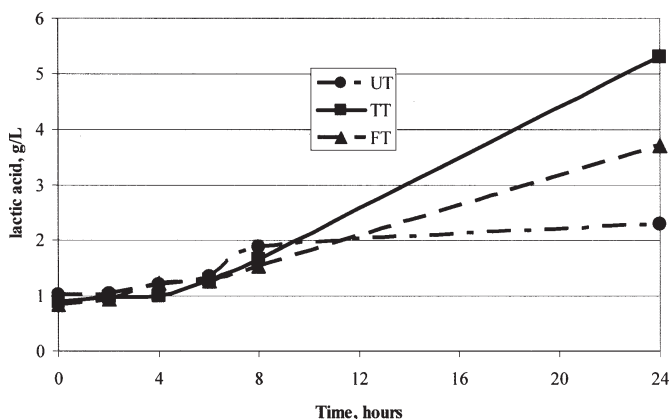


Fig. 2. Evolution of titratable acidity during the lactic acid fermentation of the cabbage juice with *Bifidobacterium* sp

fermentations by [30]. Also, the biomass/ substrate yield coefficient ($Y_{x/s}$) was higher on galactose (0.29) than on lactose (0.22), GOS (0.18), and glucose (0.15) in the case of *Bifidobacterium adolescentis* MB 239 [31].

The product yield coefficient ($Y_{p/s}$) was higher in the case of the batch thermal-treated, while the minimum value was obtained in the lactic acid fermentation of the cabbage juice without preliminary treatments before pure culture addition. [26] has established a value by 0.61 for *L. helveticus*, while 0.93 was determined by [25] in the growth of *L. lactis* NZ133 on lactose. The same parameter was calculated by [17] for fermentation of whey lactose by *Lactobacillus casei*, the values being comprised between 0.579 and 0.804g/g.

The kinetic parameters, especially the Monod constant and the maximum growth rate, are dependent on the microorganism, the nutrients that become growth limiting and also on the environmental conditions, such as temperature, pH, redox potential etc. Thus, the chemical composition of the substrate at the initial moment of fermentation could explain the differences between the determined kinetic parameters.

The correlation between the most important parameters of the lactic acid fermentation of the cabbage juices with *Bifidobacterium lactis* BB 12 were evaluated using Pearson correlation analysis ($p < 0.01$). The correlations were

moderate between glucose and viable cells, respectively glucose and biomass, while a strong relationship for glucose – lactic acid and glucose – pH can be considered (table 2).

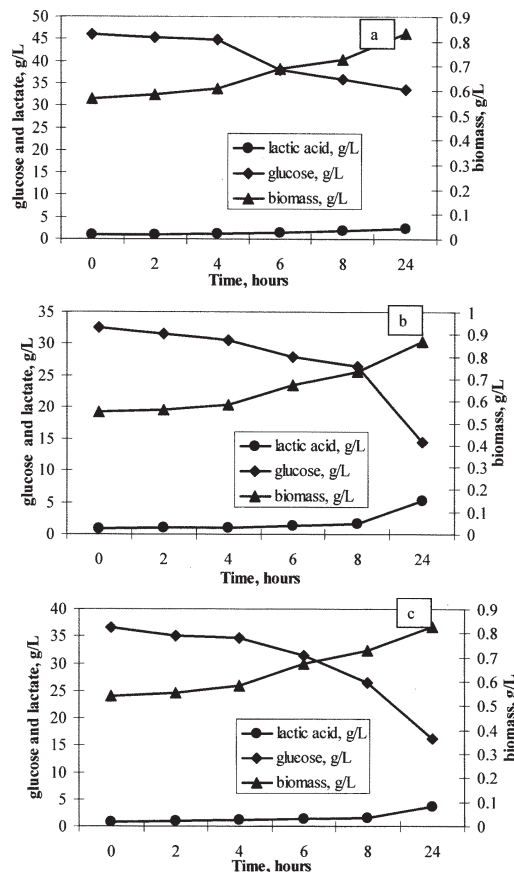


Fig. 3. Experimental data for the batches UT (a), TT (b) and FT (c)

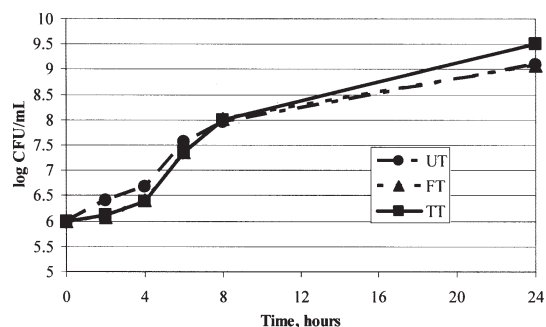


Fig. 4. Viable cell count during the lactic acid fermentation of the cabbage juice with *Bifidobacterium* sp

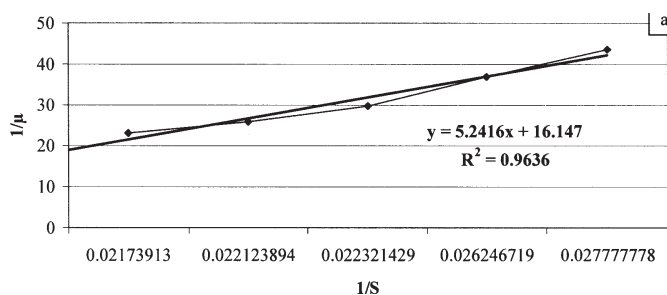


Fig. 5a. Relationship between the specific growth rate and the substrate concentration in the case of the batches UT (a)

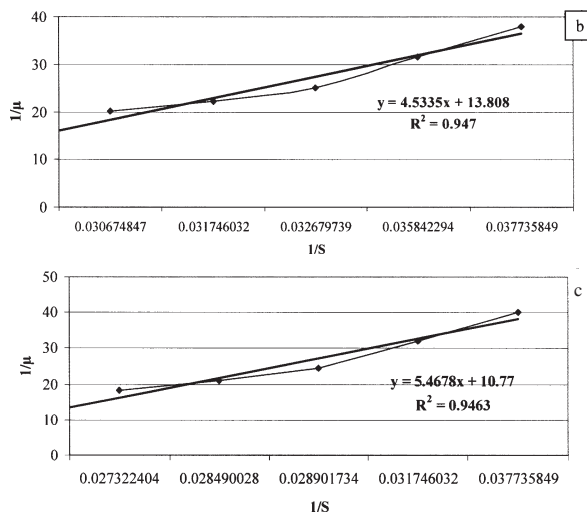


Fig. 5b,c. Relationship between the specific growth rate and the substrate concentration in the case of the batches TT (b) and FT (c)

The data scattering for the experimental batches was represented in figure 6.

Conclusions

The suitability of the cabbage juice as substrate for the growth and lactic acid production by a probiotic strain which is not present in the epiphytic microbiota of vegetables was studied. The modelling was realised applying kinetic equations for the biomass growth, the product formation and the substrate consumption.

Although the kinetic parameters were average, the cabbage juice untreated by heating, without nutrients addition, can be a favourable environment for obtaining probiotic products, with a balanced sensorial and chemical composition. In the same time, for the cabbage juice heat-treated some kinetic parameters were registered good values, from the stability point of view this one being most important.

The screening process through kinetic analysis and statistic analysis can be successfully used with a view to optimize the lactic acid fermentation and to avoid unnecessary effort in obtaining accurate values of significant parameters for the total quality of the lactic acid fermented juices.

Acknowledgement: The researches were partially performed in the frame of the Project PN-II-ID-PCE-2008-2 (ID_1359) Researches concerning the preparation of lactofermented juices with specific probiotic and evaluation through methods specific for intelligent modelation. The microorganisms were kindly provided by Chr. Hansen, Romania.

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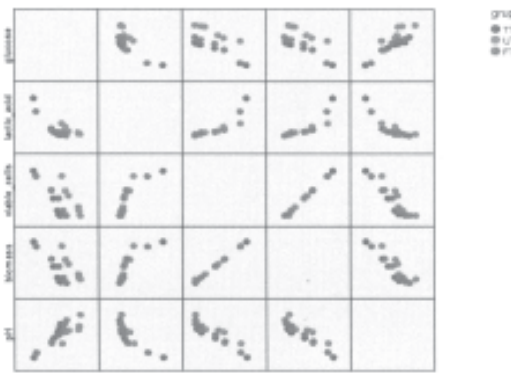


Fig.6 The scattering of the experimental data during the lactic acid fermentation of the cabbage juices with *Bifidobacterium lactis* BB 12

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Manuscript received: 16.06.2014