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Fermentation of Vegetable Juices by *Lactobacillus Acidophilus* LA-5

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Additional information is available at the end of the chapter

1. Introduction

Probiotics foods represent one of the largest sectors in functional food markets. Most of the available probiotic products are some form of dairy, despite the continuous growth of the non-dairy probiotic sector, with products like soy-based drinks, fruit-based foods, and other cereal-based products. Both non-dairy (in general) and soy-based probiotic products represent a huge growth potential for the food industry, and may be widely explored through the development of new ingredients, processes, and products. For this purpose, new studies must be carried out to: test ingredients, explore more options of media that have not yet been industrially utilized, reengineer products and processes, towards potentially meet the demands of lactose-intolerant and vegetarian consumers for new nourishing and palatable probiotic products [1].

Lactic acid bacteria are among the most important probiotic microorganisms typically associated with the human gastrointestinal tract. Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties, e.g. morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, and fermentation of various carbohydrates. However some species, like the so-called *Lactobacillus acidophilus* group and some bifidobacteria, are not readily distinguishable by phenotypic characteristics [2]. From the physiological point of view, *Lactobacillus acidophilus* strains were characterized as lactic acid bacteria with strictly homofermentative metabolism (> 85% lactic acid). The hexoses are preferential fermented via Embden – Meyerhof – Parnas (EMP), (as the strains produce aldolase and phosphoketolase), and only then the pentoses and gluconate are fermented. LAB of the *Lactobacillus acidophilus* group *as* well as of the *Bifidobacterium* group isolated from the human faeces or intestine are thought to have beneficial effects on health being thus considered to be probiotic bacteria [3].

For use in food, important criteria for probiotics must be met, in particular that they should not only be capable of surviving passage through the digestive tract, by exhibiting acid and bile tolerance, but also have the capability to proliferate in the gut.

Probiotics must be able to exert their benefits on the host through growth and/or activity in the human body. Although generally recognised as safe a probiotic strains must be characterized by a set of tests that assure its safety to consumer (1, 2, 3, 5, 6).

Inclusion of probiotic bacteria in fermented dairy products enhances their value as better therapeutic functional foods. However, insufficient viability and survival of these bacteria remain a problem in commercial food products. By selecting better functional probiotic strains and adopting improved methods to enhance survival, including the use of appropriate prebiotics and the optimal combination of probiotics and prebiotics (synbiotics), an increased delivery of viable bacteria in fermented products to the consumers can be achieved [5].

The fermentation of vegetable products, applied as a preservation method for the production of finished and half-finished food products, is considered as an important technology, though requiring more research, as a growing number of raw materials are being processed in this way by the food industry. The main reasons for this interest are nutritional, physiological and hygienic aspects of the process [6]. Thus, according to Kelwicka, (2010) [7], the fermentation of beetroot juice requires selected starter cultures made of LAB, naturally present in this vegetable although their number is usually very small. This makes them un-appropriate to, alone, conducting a fermentation that ensures satisfying sensory properties of the fermented juice, with improved health promoting activity.

Thus, probiotic juices represent an alternative to dairy products that suits consumers who don't want to eat dairy foods or are lactose intolerant. Adding probiotics to juices is more complex than formulating in dairy products where the bacteria can be easily added to other cultures.

Despite its potential for healthy products development, there is very little research activity addressing the fermentation of vegetable juices using probiotic bacteria.

2. Materials and methods

2.1. Vegetables treatments

Fresh vegetables (carrots, cucumbers, beetroot, white cabbage, red cabbage) were purchased from a retail market and specifically processed by removing the non-edible pieces. The raw material processing was made faster, because the possibility of contamination and proliferation of microorganisms in the products is very high in comparison with their intact counterparts (Lee, 2011). Using a domestic extractor the vegetables were turned into juice. The heating treatment of the juice, applied at 80^0C with a view to destroy the undesirable microorganisms under the limit of detection, was followed by cooling at 40^0C.

2.2. Microorganisms and fermentation conditions

The strain *Lactobacillus acidophilus* LA-5 from Christian Hansen (Romania) was used in this study.

The lyophilized culture was aseptically inoculated into the vegetable juices and vigorously homogenized for 15 min, according to the producer's specification. The fermentation experiments were carried out using Erlenmeyer flasks containing 50ml of juice, without pH adjustment. The flasks were incubated statically in an incubator chamber at 37 ± 0.2^0C. Sampling was taken at regular interval of times for physico-chemical and microbiological analysis.

The tested supplements were: L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany), L-lysine hydrochloride (Merck), L-valine (Merck), L-leucine (Calbiochem, San Diego, CA, USA) and yeast extract (Merck). Cysteine, lysine, valine and leucine were separately added in quantity by 0.1% (w/v) into carrot juice, while amounts by 0.2% (w/v) were tested, also individual, in the case of the yeast extract and cysteine. A control sample without supplements was carried out for each experiment.

2.3. Physico – Chemical analysis

Metabolic activity of the strain LA-5 in the conditions mentioned above was evaluated based on the dynamics of pH, respectively end products of fermentation. The pH values were measured with a HACH pH-meter. Lactic acid was determined using commercial kits (K-DLATE from Megazyme International). The calculations were made with Megazyme Mega-Calc^{TM} and expressed as g lactic acid/l. Reducing sugars were analyzed applying the spectrophotometric method with 3.5-dinitrosalicilic acid (DNS) after the removing of other substances with reducing character using basic lead acetate and expressed as g glucose/l. Ascorbic acid was determined applying the 2,6-dichloroindophenol titrimetic method, based on the reduction of the sodium salt of the dye by ascorbic acid (AOAC method). It was expressed as mg/100ml. The amino acids content, expressed as g glycine/100ml, was determined through the Sörensen method.

2.4. Microbiological analysis

The amount of viable cells of *Lactobacillus* sp. was determined by serial tenfold dilution with sterile peptone water. Aliquots of 1ml were plated, in duplicate, in plates with Man-Rogosa-Sharpe agar, enriched with L-cysteine HCl. The Petri plates were incubated for 48-72h at 37°C and the results were expressed as log colony forming units (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 juices were made. According Altiok [8], 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 100⁰C for approximately 24 h until constant weight was observed. The results were expressed as g.

2.5. Statistical analysis

Statistical analysis was carried out using the software SPSS (Statistical Package for the Social Science 17.0 trial version).

3. Results and discussions

3.1. Effect of inoculum size on the lactic acid accumulation and biomass growth

A comparative study of the dynamics of lactic acid fermentation of carrot juice using three different concentrations of lyophilized pure culture was realized (Figure 1).
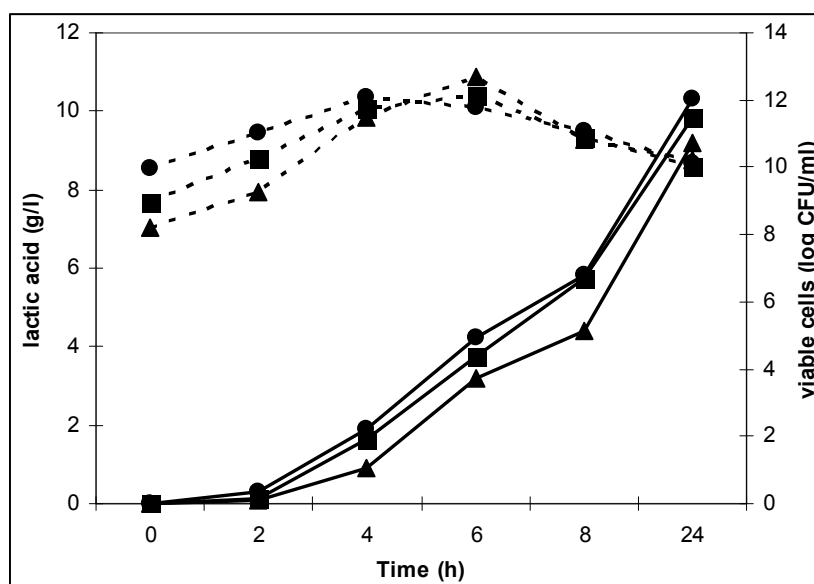

Figure 1. Correlation between lactic acid production by *Lactobacillus acidophilus* LA-5 and number of viable cells during fermentation of carrot juice with different inoculum size ▲ 0.2g/l; ■ 0.3g/l; ● 0.4 g/l (smooth lines - lactate, dashed lines - viable cells count)

Relative higher differences concerning the lactate increasing were observed between the variant with 0.2g/l pure culture initial added and the other two within 24 hours of fermentation. Thus, at the end of this interval, the excess was by 7.06% in the juice with 0.3g/l inoculum and 12.06% in the juice with 0.4g/l inoculum respectively. However, in all the batches the lactic acid accumulation, higher than 9g/l, could be considered satisfactory for the shelf life of the final products. From the other part, the number of viable cells is decisive for the probiotic feature of these ones. A direct proportionality between the amount of the lyophilised culture initial added and the viable cells was observed only in the first 4h of the fermentation. As a general characteristic, in the interval 6 - 24h pH values less than 4.5 have become inhibitory for the useful microbiota in all the experimental samples.

The initial concentration of reducing sugars of the carrot juices, by 25.2g/l, was favourable for the growth of *Lactobacillus acidophilus* LA-5. Testing two strains of Lactobacillus (one genetically selected Mont4+ and the other genetically altered, Mont4+pxyAB-mod). Kiouss [9] established that the Mont4+ had the highest yield of lactic acid fermenting with six percent concentration of glucose, whereas the L strain utilized the sugar best at the four percent concentration. In the same time temperature and pH seemed to play the largest role in the organisms ability to grow and thus affecting its production of lactic acid.

Concluding, higher inoculum densities of *Lactobacillus acidophilus* LA-5 were not significantly influenced the survival yield of the useful microbiota in the lactic acid fermented juices after 24h. In the same time, no parallel relationships between lactic acid concentration and the inoculum size were determined. The result agrees to those obtained by Agarwal, Dutt, Meghwanshi and Saxena [10] using *Enterococcus flavescens* for production of lactic acid. In their opinion, beyond a certain concentration lactic acid yield dropped due to high cell density resulting in fast depletion of essential nutrients, limiting further growth and reducing the yield. Referring to bifidobacteria, Dave and Shah [11] reported also that a higher inoculum did not always improve their viability to a satisfactory level. No data referring to *Lactobacillus acidophilus* were found in the literature.
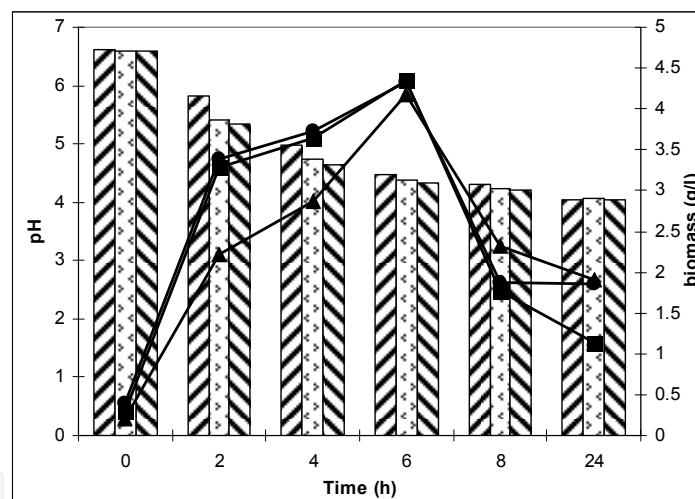

Figure 2. pH and biomass evolution during lactic acid fermentation of carrot juice with different inoculum of *Lactobacillus acidophilus* LA-5: 0.2g/l (▨ and ▲); 0.3g/l (▨ and ■); 0.4 g/l (▨ and ●); columns - pH values, lines - biomass

Although the pH dynamics was quite different in the first 6h of the process, the initial amount of the pure culture did not affect the subsequent evolution or the final value of this parameter (Figure 2).

The sharp decrease in biomass from 6 to 8h has been correlated with the viable cells tendency, as result of reaching pH values by 4.34 to 4.47. Being known that *Lb. acidophilus* is more sensitive in acidic environment, this result underlines the necessity to manage the size of inoculum in order to obtain a balance between the lactic acid accumulation and the survival of the probiotic microorganisms.

The maximum rate of acidification v_{max} was calculated as the time variation of pH (dpH/dt) and expressed as pH units/min (Table 1). Other kinetic parameters were also calculated: time to reach v_{max} (t_{max}, hours), time to reach pH 5.0 ($t_{pH\,5.0}$, hours), time to complete the fermentation ($t_{pH\,4.2}$, hours).

Inoculum, g/l	$v_{max} \cdot 10^{-3}$ (units/min.)	t_{max} (h)	$t_{pH\,5.0}$ (h)	$t_{pH\,4.2}$ (h)
0.2	7.08	4	2.95	8.4
0.3	9.83	2	2.88	8.2
0.4	10.41	2	2.67	8.05

Table 1. 1. Acidification kinetic parameters of fermentation of carrot juices by *Lactobacillus acidophilus* LA-5

A double amount of inoculum had an insignificant influence on the time to reach pH 5.0, important parameter from the shelf life of the fermented juices. Thus, $t_{pH\,5.0}$ (h) was 1.1-fold higher in the case of the batch with 0.2g/l lyophilized pure culture initial added to juice than that one with 0.4g/l. A different situation was registered concerning the maximum rate of acidification (v_{max}) and the time to reach this rate (t_{max}). Thus, a polynomial equation of the form $y = -108.5x^2 + 81.75x - 4.93$ correlated the size of inoculum with the corresponding values of v_{max} at R squared = 1. Although at the initial moment of fermentation seems to be advantageous to use a higher amount of pure culture, this aspect lessen in time, from the economic point of view being important to obtain a balance between the quantity of inoculum and the targeted parameters which ensure the preservation of the final product.

The values of the biomass content became close after about 6h of fermentation. No parallel relationship between lactic acid concentration and biomass was observed, result that agrees to those obtained by Amrane [12] and Kotzamanidis [13].

However, taking into account the lactic acid accumulation and the dynamics of the number of viable cells, it was obvious that the utilization of higher amount of inoculum is not justified.

3.2. Effect of temperature on the dynamics of fermentation

According to the information provided by the producer of the lactic culture, respectively to the data found in literature, two different incubation temperatures were tested: 37^0C and 41^0C respectively.

The dynamics of both pH and lactic acid (Figure 3) emphasizes the influence of the higher temperature on the rate of acidification. After 24h no significant differences between the pH values were determined, while the lactic acid content of the samples fermented at 41^0C was 1.24-fold higher comparatively with those fermented at 37^0C. This situation may be due to the higher amino acids content in the samples fermented at 41^0C, that act as buffer. Thus, expressed as glycin, the total amount was by 0.165g/100ml at the end of the analyzed interval, which represented an increase by 10% comparatively with the batch fermented at lower temperature.
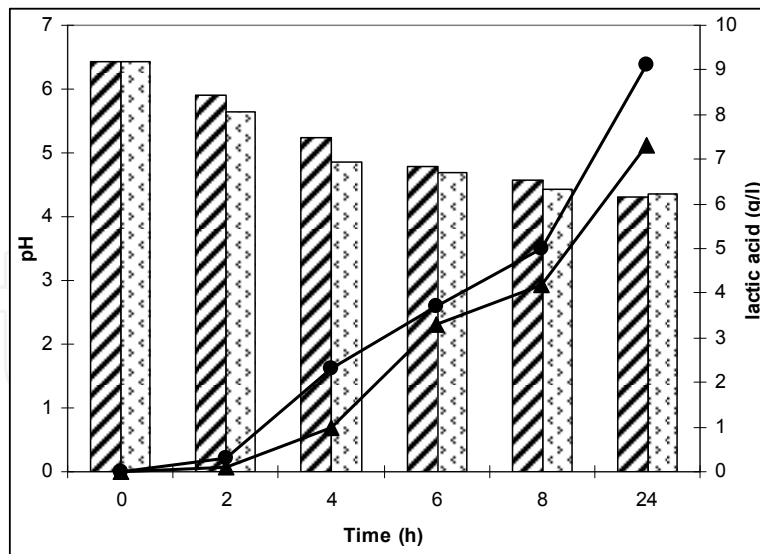
Figure 3. pH and lactic acid dynamics during the lactic acid fermentation of carrot juice at different temperatures: 37⁰C (▨ and ▲) and 41⁰C (⬚ and ●); columns - pH values, lines - lactic acid content

The rate of acidification has been correlated with the glucose consumption: 38.9% in the case of the juice fermented at 37⁰C, respectively 53.89% in the case of the juice fermented at 41⁰C. The different tendency of this parameter became obviously after 4h of fermentation (Figure 4), being the consequence of the different rate of growth of *Lactobacillus acidophilus*, expressed as optical density at 610nm.
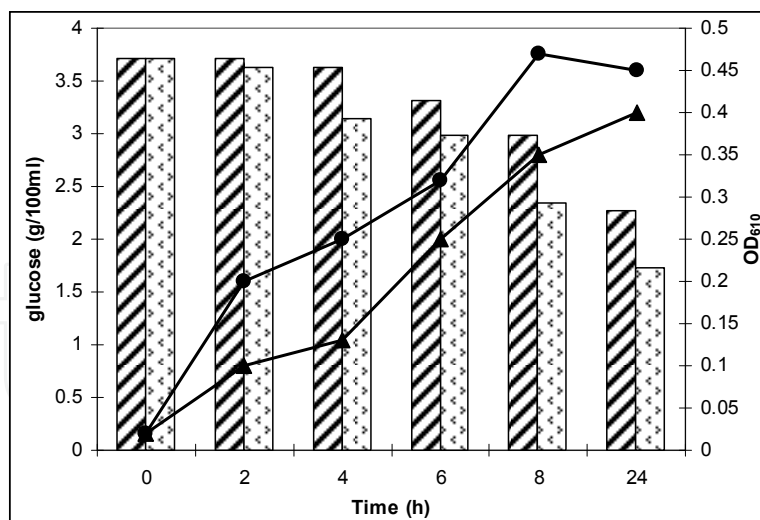

Figure 4. Glucose consumption and microbial evolution during the lactic acid fermentation of the carrot juices at different temperatures: 37⁰C (▨ and ▲) and 41⁰C (⬚ and ●); columns - glucose, lines - optical density at wavelength by 610nm

Although close, the yields of glucose conversion to lactic acid have inclined the balance in favour of the juices fermented at 37⁰C, the corresponded value being by 0.5, unlike 0.45 in the case of the juices incubated at 41⁰C.

The faster consumption of the carbon source, correlated with the growth of the useful microbiota at higher temperature, respectively with the increase of the lactic acid content until the value by 9.1g/l, was followed at 24h by the decline of the viability of *Lactobacillus acidophilus*. Taking into account the dynamics of all the above mentioned parameters, the incubation temperature applied in the further studies was by 37±0.1^{0}C.

3.3. The behaviour of different raw materials during the lactic acid fermentation by Lactobacillus acidophilus LA-5

Fresh white cabbage (*Brassica oleracea* L.), red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*), red beet (*Beta vulgaris* var. *vulgaris*), cucumbers (*Cucumis sativus*) and red onion (*Allium cepa* var. *ascalonicum*) were chosen in order to perform different experimental batches, as follows: Cb - cabbage juice, RCb - red cabbage juice, Rb - red beet juice, Cc - cucumber juice, CcO - cucumber juice with 0.1% (v/v) onion juice added after the heating and cooling of the batches.

pH and lactic acid dynamics during the lactic acid fermentation of vegetable juices with *Lb. acidophilus* are shown in Figure 5 and Figure 6 respectively. The pH values ranged from 6.29 to 3.74, no significant differences between the analyzed batches being observed, excepting the red beet juice. Thus, after one day a higher value by 4.28 was determined, the prolongation of the time of fermentation with other 24h hadn't a positive influence on this parameter.

After 24h, the highest decrease of pH was determined in the case of the cucumber juice (2.51 units), correlated with the increase of the lactic acid amount until 9.36g/l. Although the pH values of the samples Cc and Cb were close during the process development, the maximum rate of acidification v_{max} registered a better value of 9.33·10^{-3} units/min. in the case of the cucumber juice. This could explain the fermentation slowdown in the batch Cb the interval 6 - 8 hours. Correlated with the results of the microbiological analysis, it seems that this time the process was directed towards the growth of the useful microbiota. A minimum value of the maximum rate of acidification, by 6.66·10^{-3} units/min., was determined in the case of CcO, while the time to reach pH 5.0 ($t_{pH\ 5.0}$, hours) ranged between 1.9 (Cb) to 3.5 (CcO).

A relative distinct behaviour was observed in the case of red cabbage juice, red beet juice and cucumber juice with onion juice added, in the sense of the slowdown of the metabolism objectified in the dynamics of the parameters that describe the process unfolding. The differences could be explained through the presence of some chemical constituents which can act as inhibitors on useful bacteria, like anthocyanins in the red cabbage, betacyanins in red beet, respectively constituent sulfides in the onion juice. According [14], sulfides, especially those with three or more sulfur atoms, apparently possess potent antimicrobial activity. However, concerning the batch with onion juice added the initial trend was attenuated after 6 hours of fermentation, the oils and their sulfides constituent showing weak antimicrobial activity ([15]).
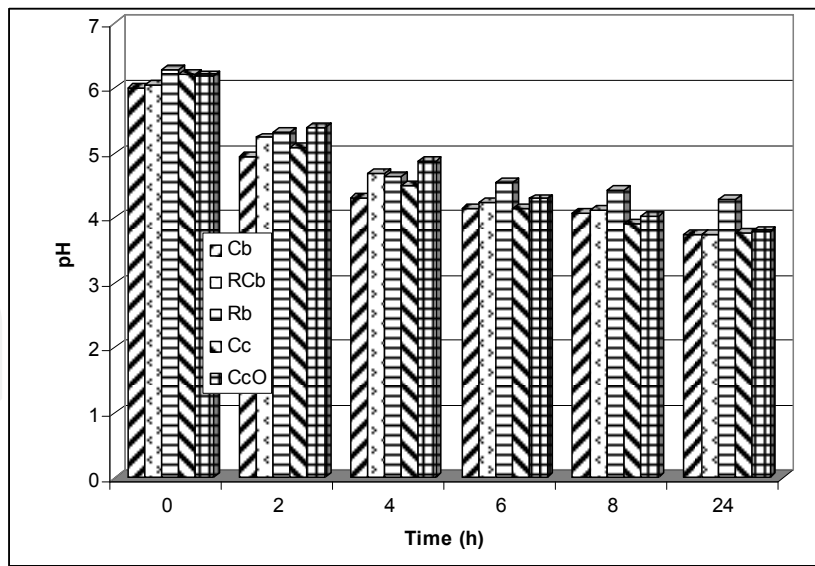
Figure 5. pH dynamics in vegetable juices obtained from different raw materials, during fermentation with *Lactobacillus acidophilus* LA-5

Referring to the red cabbage juice, although after 24 hours of fermentation the pH values were similar, the lactic acid content was lesser with about 1.5g/l compared with the white cabbage juice. This can be due to the amphoteric nature of the anthocyanins.
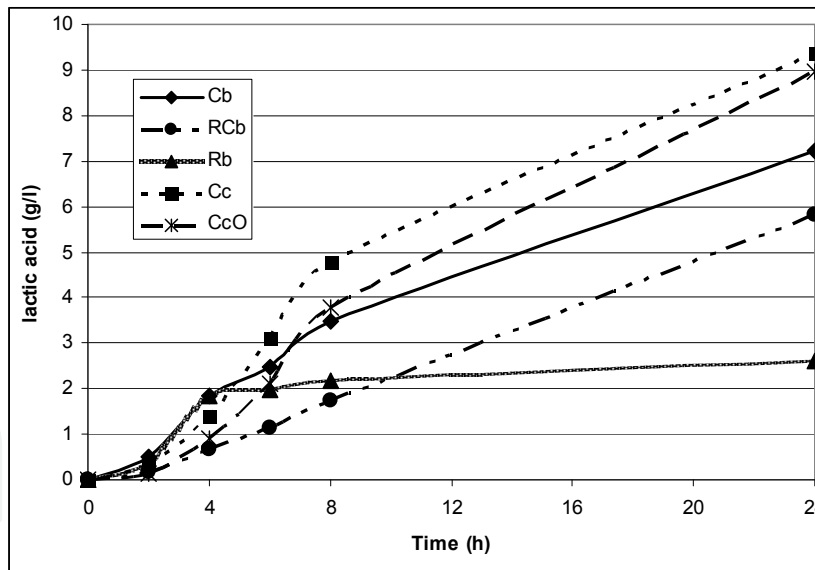

Figure 6. Lactic acid accumulation in vegetable juices obtained from different raw materials, during fermentation with *Lactobacillus acidophilus* LA-5

[16] studied the fermentation of cucumber juices with a 0.5%, 1% and 2% additions of the onion juices by *Lb. plantarum* CCM 7039. It was found that in the initial stages of fermentation, the presence of onion in the juices positively influenced lactic and acetic acid production. However, in further course of fermentation, slight inhibition effects of onion in the fermented juices were observed, especially at elevated onion/cucumber ratio.

The correlation between the biomass amount and the production of lactic acid (Figure 7) in the case of lactic acid fermentation of red beet juices with *Lactobacillus acidophilus* in the first 24 hours, was described using the Luedeking & Piret model [17]. 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), throughout fermentation at a given pH, by the expression:

$$dP/dt = \alpha \, dN/dt + \beta \, N$$

where the constants α and β are determined by the pH of the fermentation.
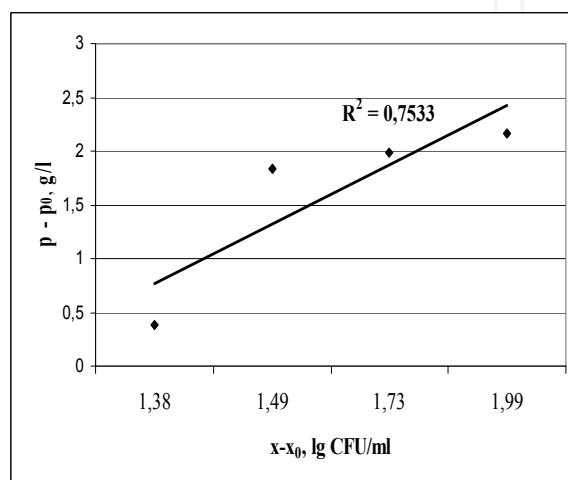

Figure 7. The correlation between the lactic acid production and viable cells count of *Lactobacillus acidophilus* LA-5 growing on red beet juices

A simplified presentation of the above model relates to the linear part of the equation which is presented as:

$$(p - p_0) = \alpha (x - x_0)$$

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 R squared coefficient closed by the ideal value ''1'' ($R^2 = 0.9989$) in the case of the carrot juices fermented with *Lactobacillus acidophilus* LA-5 (data not shown) highlights a better linear correlation, respectively a strong connection between the lactic acid production and the lactic acid bacteria growth. Not the same situation has registered in the lactic acid fermentation of the red beet juices with the same strain. The highest value of the coefficient $(1 - R^2)$ it is caused by the increase of the lactic acid amount in the first 4 hours, followed by a steady interval of evolution of this parameter. From the other hand, according [18], the deviations from the linear dependence are mostly caused by nutritive limitations of the substrates, and are related to the specific bacterial species. Not at least, the initial content of reducing sugars of the red beet, by 21.2g/l, could be limiting. However, taking into account

the fact that the cucumber juice underwent a tumultuous fermentation although its content was only with 15.09% higher, it seems that other chemical constituents of the raw materials are responsible for the above mentioned differences.

The initial content of sugars in cucumber juice was situated at the maximum limit determined by [19], while in the case of the white cabbage juice was close to that one determined by [20].
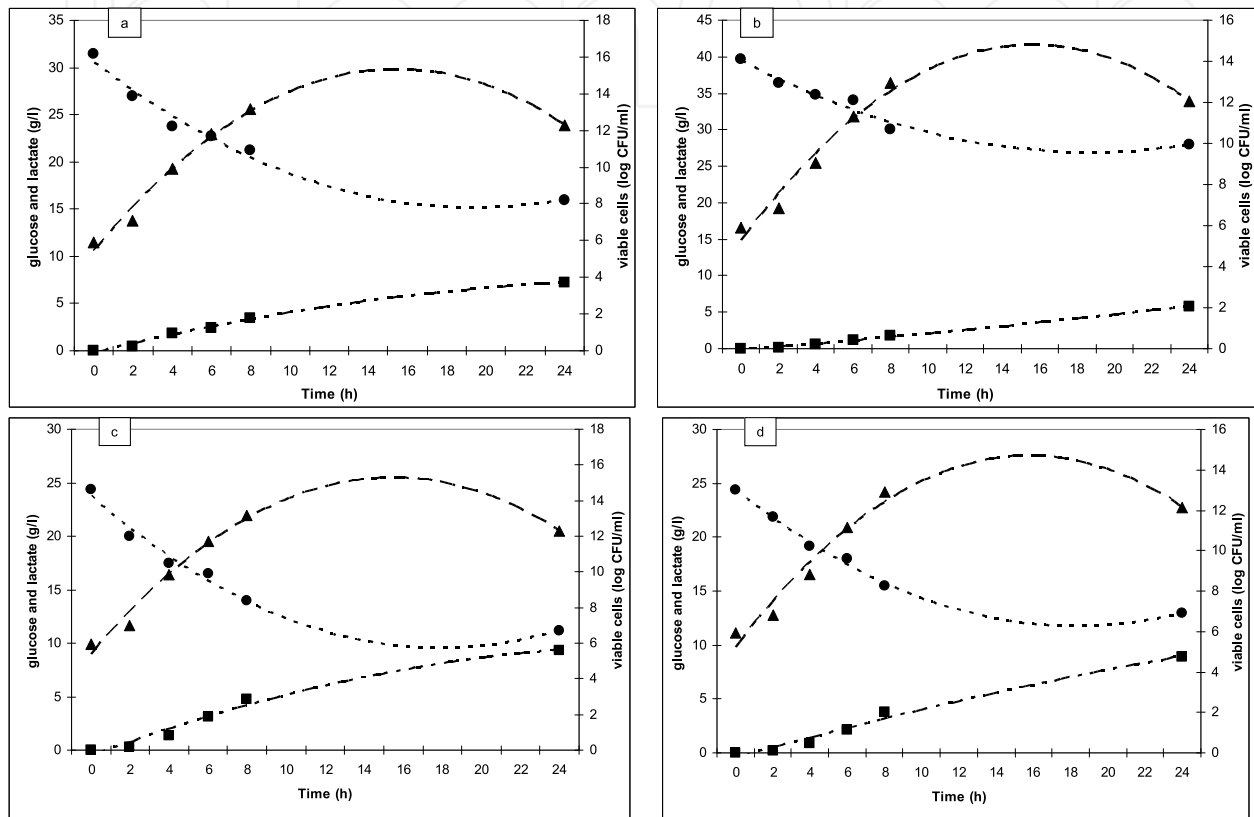

Figure 8. Correlation between the substrate consumption, lactate production and viable cells Cb (a), RCb (b), Cc (c) and CcO (d)

● - glucose, ■ - lactate, ▲ - viable cells (points - experimental data, smooth lines - predicted values)

The metabolization of the reducing sugars after 24h of lactic acid fermentation of vegetable juices with *Lb. acidophilus* LA-5 ranged between 26.66% (Rb) to 54.09% (Cc). Relative close values were obtained by other authors in lactic acid fermentation of vegetable juices. Thus, the utilization of sugar during fermentation in a mixture of beetroot juice and carrot juice and different content of brewer's yeast autolysate with *Lb. plantarum* A112 and with *Lb. acidophilus* NCDO 1748 varied from 19.4 to 24.1% ([21]).

The tested pure culture, routinely used for dairy products, was found to be capable of growing on pure vegetable juices without nutrients added. In the batches obtained from cabbage, respectively cucumber, the maximum volumetric productivity was determined after 8 hours as follows: 19.25×10^{14} CFU/(l·h) for Cb, 11.9×10^{14} CFU/(l·h) for RCb, 18.6×10^{14} CFU/(l·h) for Cc and 10.25×10^{14} CFU/(l·h) for CcO respectively.

The relationship between the growth of *Lactobacillus acidophilus*, the substrate metabolization and the lactic acid accumulation is shown in Figure 8. The prediction functions of the values of the analyzed parameters in all the samples were defined as polynomial, the R squared being very close to unit.

Correlating the number of viable cells with the dynamics of the lactic acid, the values were lower until 6 hours in the red cabbage juice and cucumber juice with onion juice added respectively. The differences were lessened in the next period of the process. However, the final yield of the lactic acid production was better in the sample CcO, by 0.78, comparatively with 0.7 in the sample Cc.

3.4. Effect of growth factors on the dynamics of the lactic acid fermentation of the carrot juices by Lactobacillus acidophilus LA-5

Kinetic parameters such as the time to reach pH 5.0 and the maximum rate of acidification are important in terms of the shelf life of the fermented vegetable juices. These ones were differently modified by the presence of the amino acids or of the yeast extract at the initial moment of fermentation. From Table 2 we deduced that a highest influence on both $t_{pH 5.0}$ and v_{max} was exerted by cysteine, added to the juice in amount by 0.2% (w/v). Compared with the other supplements, the yeast extract had a relative good effect on the analyzed parameters. At the used concentrations, the behavior of valine and lysine seems to be unobservable from this point of view, excepting the poor effect of lysine on the maximum rate of acidification. Time to complete the fermentation ($t_{pH 4.2}$, h) ranged between 7.4 (YE) and 10.42 (Leu), trend that underline the statement that in the above mentioned experimental conditions *Lactobacillus acidophilus* growing faster.

Kinetic parameter	Supplements[1]					
	Cys_1	Leu	Val	Lys	Cys_2	YE
Time-decreasing of $t_{pH 5.0}$[2]	1.28	0.85	0	0	1.69	1.1
Time-increasing of v_{max}[3]	0.82	0.84	0.98	1.05	1.1	1.05

[1]The notations used for the samples are in agreement with the nutrients added, as follows: L-Cysteine (Cys_1 sample with 0.1% cysteine and Cys_2 sample with 0.2% cysteine), L-Leucine (Leu), L-Valine (Val), L-Lysine (Lys) and yeast extract (YE) respectively
[2]The data were obtained by dividing the kinetic parameters of the control to the corresponding values of the samples
[3]The data were obtained by dividing the kinetic parameters of the samples to the corresponding values of the control
Subunit or null values should be considered as lack of effect on the analyzed parameters.

Table 2. Effect of supplements on the kinetic parameters

MRS broth used for lactobacilli enumeration often incorporates L-cysteine to improve the recovery of these ones, especially due to the fact that *Lactobacillus acidophilus* LA-5 is micro-aerophilic. Cysteine, a sulfur containing amino acid, could provide amino nitrogen as a growth factor while reducing the redox potential. [22] reported that the incubation time to reach a pH of 4.5 was greatly affected by the addition of cysteine in yogurts made with different commercial cultures, although their viability was adversely affected in function of the amount of supplement and the type of the starter culture.

Lactic acid is the major metabolite of *Lactobacillus acidophilus*, influencing both the preservation of the fermented products and the sensorial characteristics of these ones. The effect of the amino acids and of the yeast extract on the dynamics of the lactic acid, assessed against the control, is underlined through the data from Table 3. The buffering capacity of the amino acids prevented a direct proportionality between the pH values and the lactic acid content.

Time, h	Cys_1	Leu	Val	Lys	Cys_2	YE
2	8.737864	-12.6214	21.52778	-29.8611	107.6923	15.38462
4	17.66784	-23.6749	-5.55556	-2.77778	28.125	12.5
6	16.98113	-1.50943	11.71717	3.636364	1.818182	5.454545
8	20.63492	-1.5873	8.571429	1.428571	-11.1111	15.87302
24	0.925926	-0.92593	5.076142	4.568528	-14.433	11.34021

Table 3. Time-increasing of lactic acid during 24h of lactic acid fermentation of carrot juices by *Lactobacillus acidophilus* LA-5

The values were expressed in percents by reporting the difference between sample and control to the control, at the same moment of time

Negative values shows that for the corresponding interval of time the supplements had not influence on the lactic acid production at the used levels.

Analyzing the whole process, only the samples with a minimum amount of cysteine added and those with yeast extract have been a great effect on the time-increasing of lactic acid. At the other opposite were found the samples with leucine added, this amino acid with non-polar hydrophobic chains clumsying the fermentation. From the viewpoint of increase the lactic acid content in the final stages of the process, the supplementation of the carrot juices with 0.2% (w/v) cysteine seems to be undesirable.

The beneficial effect of cysteine on the lactic acid accumulation in vegetable juices can occur due to its buffering capacity, which may diminish the toxic effects of organic acids on lactobacilli. Referring to the yeast extract, which contains more cell growth factors, being used generally as a source of assimilable nitrogen, vitamins and minerals, its influence at the level of 0.2%(w/v) on the time-increasing of lactic acid could be characterized as moderate. If some authors reported different maximum lactic acid concentration in media supplemented with yeast extract, several possible explanations include the strain of microorganism, the chemical composition of the substrate, the fermentation system, and generally the conditions employed during fermentation ([12]).

Effect of supplements on the performance of lactic acid production was evaluated based on lactic acid productivity and lactic acid yield, respectively on glucose ratio (Table 4).

The previous conclusion referring to the positive influence of the yeast extract and cysteine (in minimum amount) on the development of the lactic acid fermentation of vegetable juices is confirmed by the data from Table 4. Good values of lactic acid productivity were obtained

after 24 h of fermentation in the samples with valine and lysine added, although in these ones the substrate consumption seems to be directed to the increasing of biomass, aspect emphasized by the average values of the lactic acid yield.

Parameter	Cys_1	Leu	Val	Lys	Cys_2	YE
Lactic acid yield[2)]	1.1	0.85	0.88	0.79	0.85	1.15
Lactic acid productivity[3)]	1.01	0.99	1.06	1.05	0.7	1.13
Glucose conversion ratio[4)]	1.1	0.9	1.2	1.05	0.92	1.25

[1)]The data from the table were obtained by dividing the corresponding values for the samples to those of the control
[2)]Lactic acid yield was calculated by dividing the amount of lactic acid produced to the amount of glucose consumed
[3)]Lactic acid productivity was defined as the amount of lactic acid produced per hour per liter
[4)]Glucose conversion ratio was calculated by dividing the amount of glucose consumed to the initial amount of glucose.

Table 4. Effect of supplements on lactic/acetic acid production after 48 h of fermentation[1)]

The effect of supplements (amino acids and yeast extract) on the ascorbic acid dynamics is shown in Figure 9. L-Ascorbic acid (AA), also known as vitamin C, is a representative water-soluble vitamin possessing a variety of biological, pharmaceutical, and dermatological functions; it promotes collagen biosynthesis, provides photoprotection, causes melanin reduction, scavenges free radicals, and enhances immunity ([23]).

Due to the heat treatment applied with a view to destroy the epiphytic microbiota of the fresh vegetable juices, the losses occurred in the ascorbic acid content represented about 65%.
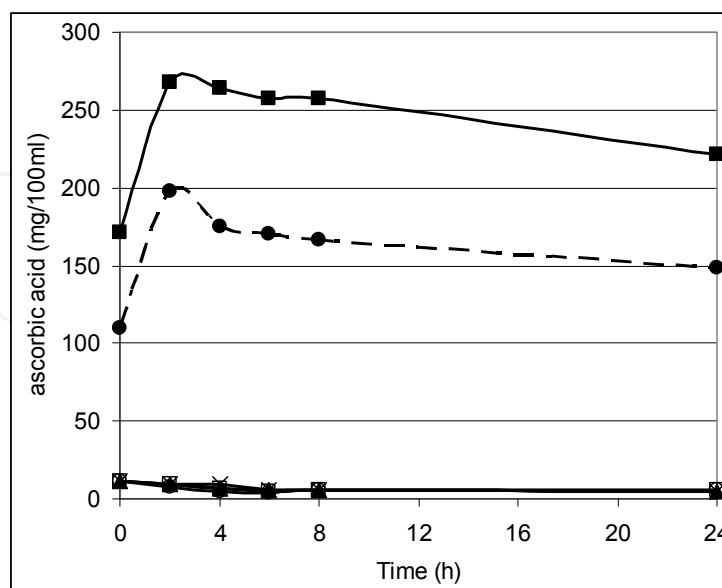

Figure 9. Time-course (0-24h) of the relative levels of ascorbic acid
(●Cys_1, ■Cys_2, ○YE, □Leu, ▲Val, x Lis). The data shown are average values of two independent replicate experiments

The presence of ascorbic acid into vegetable juices submitted to fermentation by probiotic bacteria, especially by *Lactobacillus acidophilus* strains, is desired not only from the nutritional point of view, but also due to the fact that it could promote anaerobic conditions, acting as an oxygen scavenger. [24] have shown also that the fruit juices may be an alternative vehicle for the incorporation of probiotics because they are rich in nutrients and do not contain starter cultures that compete for nutrients with probiotics. Furthermore, fruit juices are often supplemented with oxygen scavenging ingredients such as ascorbic acid, thus promoting anaerobic conditions.

L-cysteine, a sulfur-containing amino acid known as a powerful reducing agent, caused the reduction of dehydroascorbic acid to ascorbic acid, which led a different behavior of the samples Cys_1 and Cys_2 by the others. The increase of this parameter was by 80% and 56.4% respectively, after 2h from the initial moment of fermentation. Subsequently, the analyzed parameter had the same diminishing tendency as in the other batches.

The losses occurred after 24h of lactic acid fermentation of carrot juices with *Lactobacillus acidophilus* LA-5 ranged from 48.39% (YE) to 61.9% (control). The possible reason could be the oxygen traces that cause the chemical oxidation of the vitamin C.

In order to evaluate the probiotic feature of the vegetable juices, the study of the effect of supplements on *Lactobacillus acidophilus* growth is from overwhelming importance, both during the lactic acid fermentation and during the storage of the final products.

Between the analyzed samples, those with yeast extract and 0.1% (w/v) cysteine added registered a higher increase of the number of viable cells till 14.4 - 14.5 log CFU/ml in the first 8h of the process. Concerning the yeast extract, the most possible explanation is due to an enhanced availability of minerals, which are growth promoters for *L. acidophilus* ([25]), while discussing the factors that affect the activity of endogenous probiotics, (26) mentioned that some of the growth promoters in cow milk were apparently cysteine-containing peptides.

Referring to the juices with leucine, lower values were determined comparative with the control during 24h, while in the samples with 0.2% (w/v) cysteine added the trend of the survival of lactobacilli was slow down in the period 6 - 8h, the level being by 13.5 and 13.6 log CFU/ml respectively. The last observation agrees with this one of [27], which have shown that the increasing of cysteine concentration improved the viability of *B. bifidum* in bio-yogurt, although it had no important effect on the viability of *Lactobacillus acidophilus*.

The batches supplemented with valine and lysine had occupied an intermediate position, the growth until 14.2 log CFU/ml after 8h of fermentation making from the utilization of these amino acids a promising variant in the future, with a view to optimize the conditions of the process unfolding. In the period 8 - 24h the number of viable cells decreased, as result of the lack of tolerance at lower pH of the analyzed strain.

The correlation between the most important parameters of the lactic acid fermentation of the carrot juices with *Lactobacillus acidophilus* LA-5 were evaluated using Pearson correlation analysis (significance level *p* < 0.01; confidence level of 99%).

Analytical variables	pH	lactic acid	glucose	viable cells	glycine	ascorbic acid
pH	1	-0.889**	0.829**	-0.940**	0.099*	-0.184*
lactic acid		1	-0.891**	0.843**	-0.201*	0.016*
glucose			1	-0.789**	0.093*	0.084*
viable cells				1	-0.061*	0.066*
glycine					1	-0.103*
ascorbic acid						1

** Correlation is significant at the 0.01 level (2-tailed)
* Not significant

Table 5. The Pearson coefficients for the experimental batches

The correlations are strong between pH and lactic acid, respectively pH and glucose, while a very strong relationship pH - viable cells could be considered (Table 5). A non-existent relationship between ascorbic acid / amino acids content (expressed as glycine) and the other analyzed parameters was determined.

A firm correlation between glucose and lactic acid was expected, but on the one hand it is known that the practical yield of sugars conversion to lactic acid of the strains of the group *Lb. acidophilus* is about 85%, while on the other hand the analysis does not include supplementary data referring to other factors that might be involved in the dynamics of the lactic acid fermentation of vegetable juices.

Factor Analysis (FA) is a multidimensional statistic method whose purpose is the analysis of the structure of mutual dependences of variables. The method is similar to the Principal Component Analysis (PCA) with the exception of the factor weights that are scaled ([28]).

Applying FA to the experimental data, the analytical variables were reduced to two principal components, which accounted for 59.72% (PC1) and respectively 18.95% (PC2) from the total variance. According to the component matrix, respectively to the values of the component loadings expressed by the first second principal components (rotation method: Varimax with Kaiser normalization), the most notable variables were pH and lactic acid (equal loading values by 0.954). Higher values were obtained also for viable cells (loading 0.939) and glucose (loading 0.933).

The combination of PC1 and PC2 (Figure 10) underlined the lack of correlation between amino acids content / ascorbic acid and all the other parameters taking into account both control and supplemented samples. While PC1 affected the dependent and independent variables involved in the progress of the lactic acid fermentation of vegetable juices, respectively in their probiotic feature, PC2 separated the variables which contribute to the nutritional characteristics of the final products.
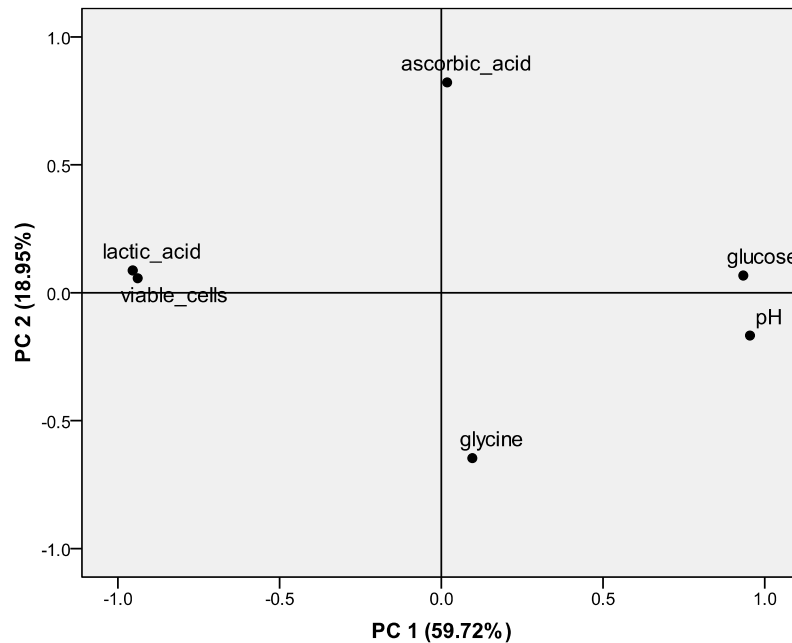
Figure 10. Component plot in rotated space

Applying PCA to the lactic acid fermentation of cabbage juices with various microorganisms, [29] established that the original 7 analytical variables were reduced also to 2 independent components that explained 88.2% from total variance of input data (PC1 66.9% and PC2 21.3%).

Cluster Analysis (CA) is a statistic method whose purpose is to join data into clusters with a view to increase their withingroup homogeneity. Usually, the FA is considered the first step of CA, with a view to reduce the data dimensionality. In order to better distinguish among experimental samples, the cluster method of the nearest neighbour was used. The distances between objects were measured as squared Euclidean distance. K-Means Cluster Analysis divided the experimental data into three groups, characterized by similar analytical properties, as follows:

- cluster 1: all the carrot juices (control samples and the batches with amino acids and yeast extract added) at the initial moment of fermentation, respectively at 2th h of fermentation. Supplementary, this cluster included the control and the sample with leucine at 4th h of fermentation (C_4 and Leu_4);
- cluster 2: all the carrot juices at 24th h of fermentation and the sample with lysine added at 8th h of fermentation ;
- cluster 3: the carrot juices with leucine and lysine added, respectively the control, at 6th and 8th h of fermentation (Leu_6, Leu_8, Lys_6, Lys_8, C_6, C_8); the carrot juices with cysteine, valine, respectively yeast extract from 4th to 8th h of fermentation (Cys_4 - Cys_8, (Val_4 - Val_8), (YE_4 - YE_8).
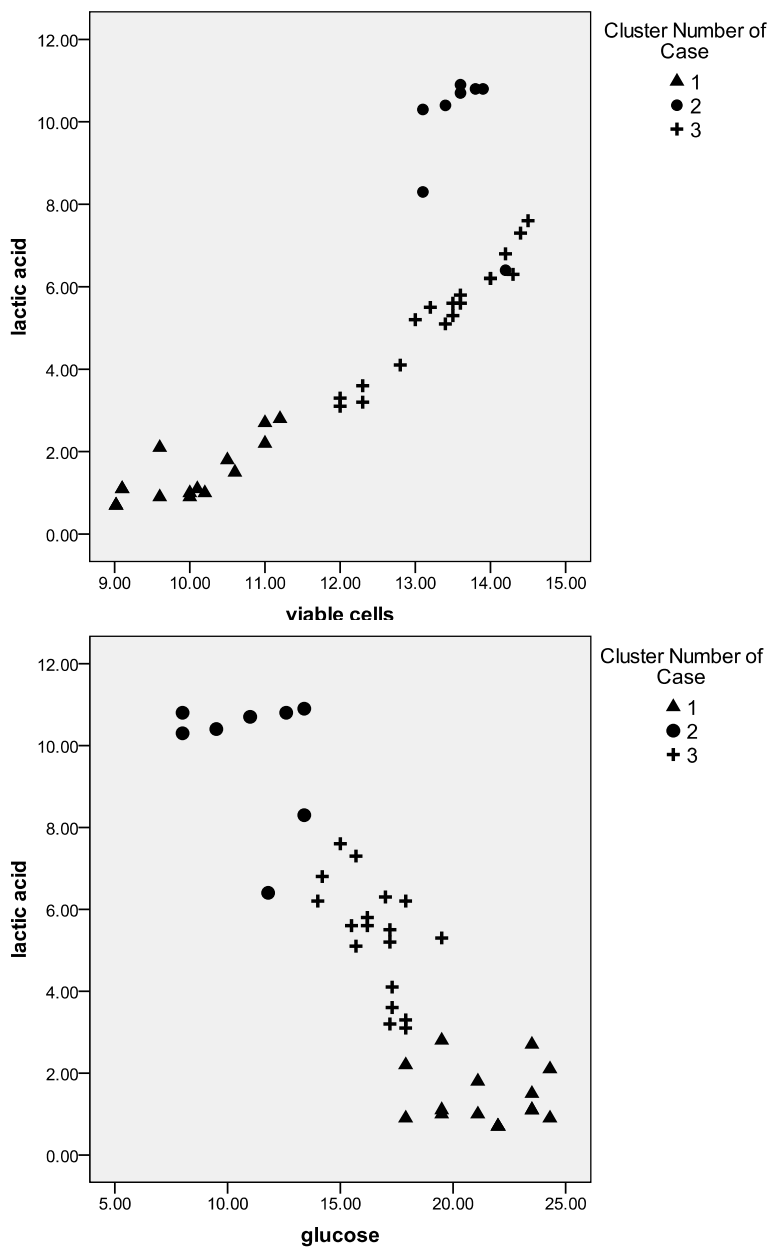
Figure 11. Clusters plotting in coordinate of two selected variables: lactic acid - viable cells and lactic acid - glucose

The clusters in axes of two selected variables (Figure 11) denote that the samples from the first cluster were marked with a higher content of substrate, null or very lower lactate amount and pH values more than 5. The corresponding time was both 0 and 2h (C, Val, Leu, and YE) or the entire interval 0 - 4h (Leu and control).

The samples at the final moment of fermentation and those with lysine after 8h of the process were included in the second cluster, characterized through lower or average values of glucose content, higher lactic acid amount and pH values close to 4.2. This cluster marks the achievement of the optimum characteristics of the lactic acid fermented products.

The samples included in the third cluster best describes a vigorous process, being characterized through average values of the main parameters involved in the dynamics of the lactic acid fermentation of vegetable juices.

The usefulness of the methods of statistical analysis is underlined by a lot of applications of CA that could be reported: in evaluation of analytical and sensory characteristics of vegetable juices ([28], [29]), in distinguishing between wines aged a different number of months ([30]).

4. Further research

The importance of consuming probiotic foods for the improvement of the quality of life increasingly more in the last years, being underlined by the scientific literature. The diversification of the market from this point of view could be strong correlated with the increasing of the life expectancy worldwide.

Our further researches are needed in order to optimize the level of nutrients (individually and in combination) and in the same time their influence on growth and viability of probiotics (in particular of *Lactobacillus acidophilus*, single strain or in combination with other probiotics), not only during fermentation but especially during the storage of the final products.

5. Conclusions

Different vegetable juices are suitable and alternative food matrices for the production of functional foods with *Lactobacillus acidophilus* LA-5, a probiotic strain which is not present in the epiphytic microbiota. Although some differences between the growths trends were determined, all the analyzed vegetables could be considered proper in order to obtain lactic acid fermented juices with a higher self-life. Application of Principal Component Analysis selected the most important parameters from analytical point of view: pH, lactic acid, biomass and viable cells, while the Cluster Analysis divided the experimental variables into three groups.

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