Phenotypical characterization of several lactic acid bacteria strains isolated from wheat's epiphyte microbiota

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Abstract

Five new strains of Lactobacillus sp. have been isolated from wheat epiphyte microbiota using an original method and stored in the laboratory collection at -70℃ in appropriate medium represented by MRS broth (Merck, Darmstadt, Germany) supplemented with 20% (v/v) glycerol. All strains were phenotypical characterized (microbiological, biochemical and biotechnological): optimal temperature, effect of sodium chloride and pH during growth, type of fermentation, auxogram, production of polysaccharides, ability to hydrolyze the starch, arginine and esculine hydrolysis, resistance to billiary salts, production of H_2S, reduction of the tetrazolium salts, production of oxygenated water, production of catalase, Voges Proskauer reaction, etc. All strains were identified with API 50CH microtest systems from BioMerieux (Lyon). Thus: 13, 14, 15 – GAL are Lb. plantarum, and 16, 17 – GAL are Lb. brevis.

Keywords: *Lactobacillus*, isolation, identification, preservation, viability.

Introduction

Diversity and severity of diseases specific to current civilization, the reduced immunity of modern human-being have driven researchers to increasingly study the food – medicine relation thoroughly in preventing or mending some diseases [1].
Lactic acid bacteria (LAB) are considered traditional probiotics. They are used from very ancient times especially in milk processing firstly thanks to their fermentative properties, but also as a protective and safety factor (towards deterioration). Probiotics can be defined as alive microorganisms that after being eaten in a certain number, bring benefits to consumer`s health apart from their whole nutritional quality of the food where they could be found [2,3,4,5,6,7].
Thanks to the commercial interest for functional food containing probiotics, the scientific interests in these products has also increased. Lately, the researches in the food biotechnologies are centered on careful isolation and selection of new "safety" strains of *Lactobacillus* that could ensure the microbiological security of the food and bring benefits to the consumer`s health [2,3]. Before these strains are being turned to the best by the food industry they are microbiologically, biochemical and genetically characterized on the basis of sophisticated molecular researches [8, 9,10]. A distinctive feature of lactic acid bacteria (LAB) is that their growth is accompanied by lactic acid production, which results in acidification of the medium and, eventually, transition to stationary phase. For eubacteria, an acid environment inhibits cell multiplication and can result in cell death. As their own growth

results in an acidic environment, LAB would be expected to have efficient acid stress resistance mechanisms to allow their survival [10].
Properties such as: tolerance to bile salts and low gastrointestinal *p*H, the colonization and adhesion capacity of the intestinal tract, the antimicrobial activity through bacteriocine and lactic acid production, thermoresistance that could adapt itself to the technological conditions of processing the product as well as the complex visceral ecosystem, the survival rate during the starter culture storing and the food product are only some of the biotechnological researchers preoccupations nowadays [11,12].
The aim of this work is to isolate, to identificate and to characterize the phenotyp of several new lactic acid bacteria strains from wheat epiphyte microbiota.

Materials and Methods

1. Isolation of lactic acid bacteria

The lactic acid bacteria strains 13 – 17 GAL were isolated from wheat epiphytic microbiota using the original method following:

- The enrichment stage consists in the aseptic milling of the wheat sample with a glucose solution (10g glucose/100ml) mixed in a 2:1 ratio (w/v), up to breaking the grains and making a paste. It is then thermostated in conditions of anaerobiosis at a temperature of 37°C, for 18 – 24h. Lactic acid bacteria develops, as a result of conditions created and lactic fermentation takes place thanks to its access to the glucidic sublayer in the caryopsis endosperm.
- Inoculation by the double layer method. After incubation, aseptic probes are taken from the sample and dilutions are carried out. From these dilutions, 100 μl are inoculated on solid MRS medium with 1.5% (w/v) agar and 1% (w/v) $CaCO_3$. It is spread with a Drigalski spatula until the suspension is adsorbed. Then it is poured in each plate, 4 – 5 ml half solid MRS with 0.75% (w/v) agar and 1% (w/v) $CaCO_3$ and it is incubated at 37°C, for 24-48 hours.
- Isolation of pure culture is carried out by spreading: the scarified or the Koch methods [12].

All strains were stored in the laboratory collection at -70°C in appropriate medium represented by broth MRS supplemented with 20% (v/v) glycerol. Primary cultures were obtained and after cultivation on MRS broth in order to obtain mid-logarithmic phase cultures that were further used in our experiments.

2. Phenotypical characterization methods of the lactic acid bacteria strains

- The optimal temperature for development: The isolated strains have been inoculated on the MRS broth and incubated at 5°C, 15°C, 28°C, 37°C, 42°C, 45°C, 48°C and 60°C. The analysis done spectrophotometrically – measuring the OD_{600} values at 12h and 24h, this indicates the degree of increase and growth.
- The effect of sodium chloride upon the increase and growth: it has been estimated through the inoculation of the isolated strains on APT medium which NaCl in variable concentration of 2, 4, 6, 8, 10% (w/v) is added. The tubes have been incubated at 37°C and OD_{600} at 12h and 24h has been determined in order to estimate the increase and growth of pure clones in the circumstances imposed by salinity.
- The effect of *p*H upon the increase and growth: has been determined on MRS adjusted after the sterilization of the medium at *p*H: 3.0, 3.9, 5.5 with sterile acetic acid at *p*H: 6.5, 7.0, 7.5, 8.0 with NaOH 1N. The tubes have been incubated at 37°C and OD_{600} at 12h and

24h has been determined to estimate the increase and growth of the pure clones in the conditions imposed by *p*H. [13]

- The type of fermentation: has been determined using the liquid medium Mac Cleskey (g/l): triptone 16, yeast extract 8, bipotassic phosphate 1.5, glucose 80, *p*H 7.0. After inoculation 4 ml of „white" gelose (15 g agar at 1 L of distilled water) are added in each test tube. The incubation is done at 37°C and the test tubes are being watched for 2 – 7 days.
- The ability of metabolizing simple glucides – auxograme: was made on a breeding medium (g/l) with peptone 10, NaCl 5, bromtymol blue under the form of an alcoholic solution 0,06; the substratum prepared under the form of a solution 10% (v/v), separately sterilized, was added in a proportion of 1% (v/v). The following carbohydrates have been used: lactose, glucose, mannose, galactose, maltose, saccharose, ribose, arabinose, dextrine, xylose, fructose, raphinose. The incubation has been done for 48h at 37°C, but the tubes have been watched 7 – 10 days in order to estimate the growth.
- The ability to hydrolyze the starch: has been achieved on medium with the following composition (g/l): peptone 15, NaCl 5, yeast extract 6, galactose 5, starch 2, Tween 80 - 1ml, agar 20g/l. Plates were incubated at 37°C for 2 to 8 days marking the date when each of them has got positive.
- The production of polysaccharides: has been achieved on a medium with the following composition (g/l): peptone 5, extract of meat 10, NaCl 5, potassium nitrate 8, saccharose 8, tween 80 - 1 ml. After cultivation at 37°C for 2 to 8 days the probes are centrifuged for 10 minutes, at 5000 rot/min. An equal volume of ethanol 95% (v/v) is added to the supernatant.
- The production of H_2S: is made evident on a medium with (g/l) peptone 15, casein 5, glucose 1, acetate of lead 0.2, sodium tyosulphate 0.08, manganese sulphate 0.05, Tween 80 -1 ml, agar 15. The inoculated tubes are incubated at 37°C for 2 weeks.
- The hydrolysis of arginine: can be made evident on MRS with arginine 0.3% (w/v). It is incubated 2 – 3 days at 37°C. The excess ammonia is eliminated in the medium and determines the appearance of a yellow orange precipitate as a result of a color reaction with Nessler reagent.
- The hydrolysis of esculine: is made evident on a medium with the following composition (g/l): peptone from casein 10, yeast extract 5, sodium acetate 5, Tween 80 - 1 ml, manganese sulphate, esculine 5, ammoniacal ferum citrate 0.5, pH 6.5. The incubation takes place at 37°C for 2 to 3 days.
- The resistance to billiary salts: A medium containing tomato juice (g/l): 100, peptone 15, glucose 20, NaCl 5, Tween 80 -1 ml, yeast extract 6, soluble starch 0.5, tauroglicocolate 20 is achieved. The incubation is done at 37°C for 7 to 8 days.
- The reduction of the tetrazolium salts: is made evident on a medium with the following composition (g/l): peptone 15, yeast extract 1, Tween 80-1 ml, dipotassic phosphate, magnesium sulphate 0.05, agar 10. After the autoclaving 0.1% 2,3,5 triphenil tetrazolium chloride (TTC) is added as a redox indicator.
- The production of oxygenated water: is made evident on manganese gelose glucose oxide with the following composition (g/l): peptone 5, extract of meat 5, yeast extract 5, glucose 5, Tween 80 - 0.5, manganese sulphate 0.1, agar 15, *p*H 7.0. After the allocation in plates it is covered with a thin layer of manganese oxide 4% (w/v).
- The production of catalase: is verified through the depositing of several drops of oxygenated water on the surface of colonies on agarised MRS.

- Voges Proskauer reaction: we add 5 ml solution NaOH to 5 ml bacterial culture. In order to hasten the reaction we add 5 ml of bacterial culture, 2 drops of solution $FeCl_3$ 2% and then other 5 ml solution NaOH 10% (w/v). It is left at 37°C for 1 h.

3. Strains identification

Identification of selected strains is a major step prior to their full scale use. The BioMerieux Company sells standardised, quick biochemical tests that ensure an accurate taxonomic determination of isolated strains. API 50 CHL Medium, intended for the correctly identification of the genus *Lactobacillus* and related genera, is a ready-to-use medium which allows the fermentation of the 49 carbohydrates on the API 50 CH strip. API 20 Strep is a standardized system and consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. It enables species identification of most streptococci and enterococci, and those most common related microorganisms.

Each strain was analysed with the API 50CH test, and 14GAL was simultaneously determinate with the API 20 Strep test as well, because there were doubts following the phenotypical analysis carried out on multiple phase taxonomy criteria and even as a result of the molecular genetic surveys done formerly.

4. Biotechnological characterization of isolated lactic acid bacteria

4.1. Multiplication dynamics of isolated lactic acid bacteria in discontinuous culture

In order to draw the development curves for isolated lactic acid strains the following parameters have been tracked: *p*H, acidity, OD_{600}, CFU/ml. For each strain, 14 tubes were inoculated with 10 ml MRS with 0.1 ml pure culture and were incubated at 37°C a variable time – from 2h to 48h. Every 2 hours, up to 24h and then 48h, the required parameters were determined.

- As a result of metabolic activity, lactic acid bacteria changed the *p*H of the culture medium particularly by producing lactic acid, and this modification is correlated with cultivation.
- Determination of acidity. Acidity is expressed in percentage of sulphuric acid and is calculated with the following formula:

  $$\text{Acidity } (H_2SO_4) = \frac{0{,}0049 \cdot V_2}{V_1} \cdot 100 \qquad (\%)$$

  0.0049 = amount of sulphuric acid expressed in grams equivalent to 1 ml solution of 0.1N NaOH; V_1 = sample volume (10ml); V_2 = volume (ml) of NaOH solution 0.1N used for titration.
- Determination of CFU/ml (colony formation units) was carried out by indirect cultural methods, by inoculation on MRS (in double layer), based on the formula:
  $CFU/ml = 10 \cdot n \cdot k$;
  n = number of colonies
  k = dilution coefficient suitable to the plate where counting was done.
- Another tracked parameter is optical density at the wave length λ = 600 nm (OD_{600}) as it is known that between the culture absorbance and cell concentration in suspension, there is a similar proportionality with the Beer-Lambert law, according to relation: $A = \varepsilon \cdot I \cdot C$;
  A = absorbance measured at λ = 600 nm,
  ε = extinction coefficient,
  C = cell concentration in suspension. According to the dry substance of cellular mass and culture absorbance, compared to the sterile culture medium considered as a reference, a reference curve A_{600}= f (dry substance biomass) can be traced.

4.3. Determination of pure *Lactobacillus* culture viability during freezing

Verification of the preserved culture viability has been done periodically because freezing as well as liofilization, tense microorganisms. Thus, CFU/ml was determined for each strain, by growing on MRS in double layer: before and after freezing, at 24h-, at 3, 6, 9 months and 1 year.

Results and Discussion

1. Isolation of lactic acid bacteria from wheat microbiota

By inducing a lactic acid fermentation of wheat grains, from the epiphytic microbiota, the lactic acid bacteria colonies have been selectively distinguished, by cultivation on MRS with 1% $CaCO_3$ (w/v) in double layer. These present around them a clear halo, while the rest of the medium in the plate is opalescent (fig.1). Thus, the lactic acid bacteria show small, round, colonies, having 1 – 2mm diameter, some of them are slightly, others are lenticular, with a round perimeter, smooth, white-cream colored, some are slightly fluorescent (fig.1), colonial characters that distinguish from the colonies of other microorganisms in the product's specific microbiota.

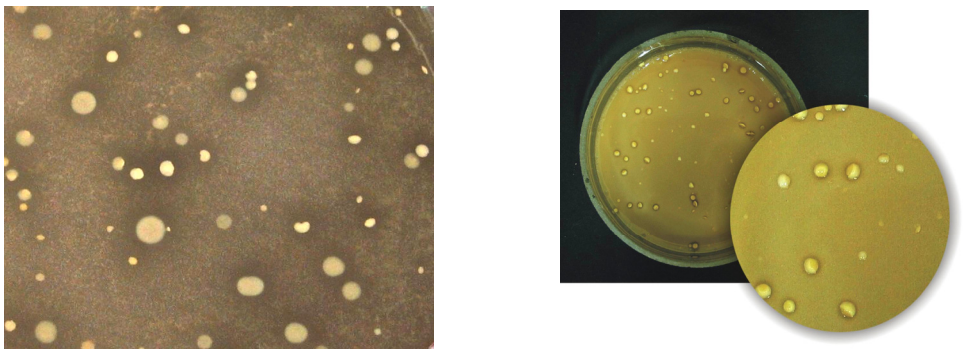


Figure 1. Culture characters of *Lactobacillus* sp. strains on MRS medium containing 1% $CaCO_3$

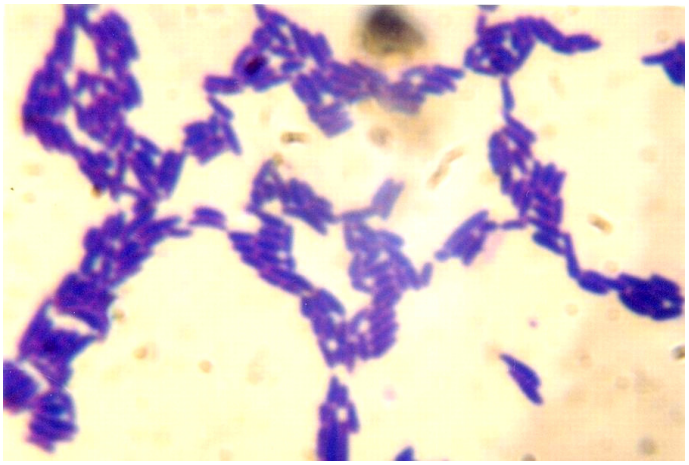


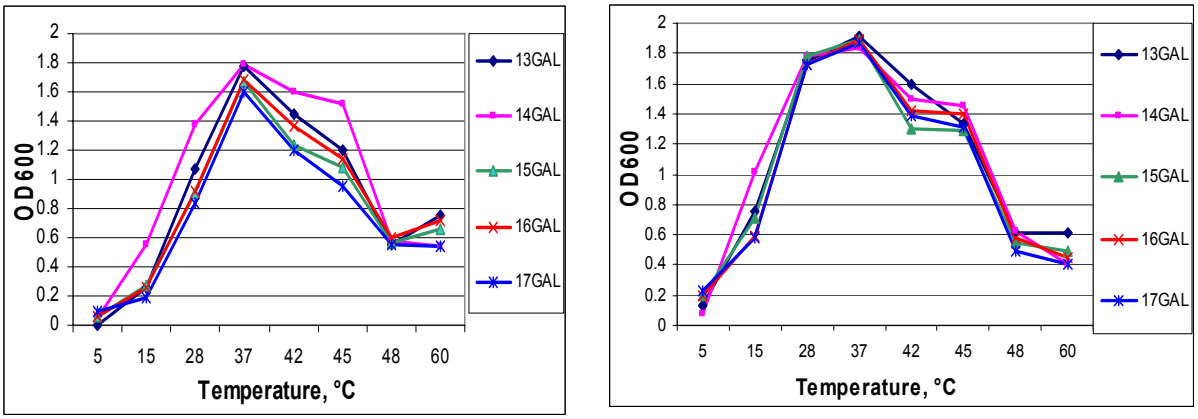
Figure 2. Microscopic aspect of *Lactobacillus* sp. 16 GAL strain (at immersion objectif with magnification 100X)

After preparing the microscopic slides to certify the existence of the Gram-positive lactic bacteria, (lactococci arranged in chains of different sizes and lactobacilli of various sizes

isolated or arranged in short chains or in palisade) (fig.2), their isolation was carried out in pure cultures in MRS broth. Five lactic acid bacteria were isolated from the coded wheat epiphytic microbiota: 13GAL, 14GAL, 15GAL, 16GAL, 17GAL.

2. Phenotypical characterization of the lactic acid bacteria strains

All the strains of *Lactobacillus* sp. studied grow in a temperature interval of 28°C to 45°C the optimum temperature being 37°C at 12h and 24 h. The strains record higher values of OD_{600} after 24 h and at 28°C, but it develops faster (at 12 h) at 45°C than at 28°C (fig.3).



a b

Figure 3. Growth of *Lactobacillus* strains at different temperature values (OD_{600}) at 12h (a) and 24h (b)

All the strains of *Lactobacillus sp.* studied grow well at concentrations of 2 – 4% (w/v) NaCl in medium and not prefer a higher salinity (fig.4).

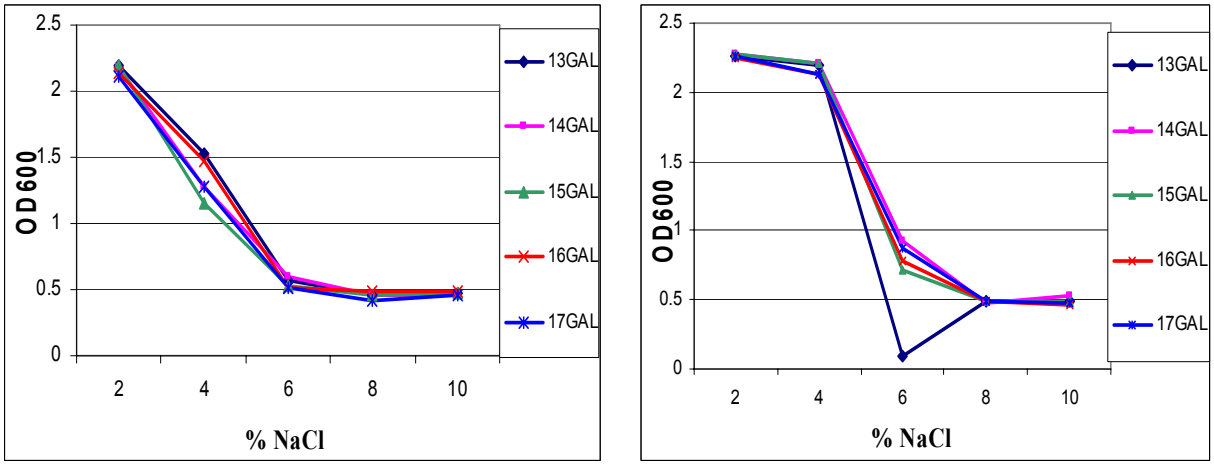


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Figure 4. Growth of the strains at different NaCl concentrations (OD_{600}) at 12h (a) and 24h (b)

The domain of *p*H in which the isolated strains grow is 5.5 – 7.0, but the optimum is at *p*H 7.0 (fig.5).

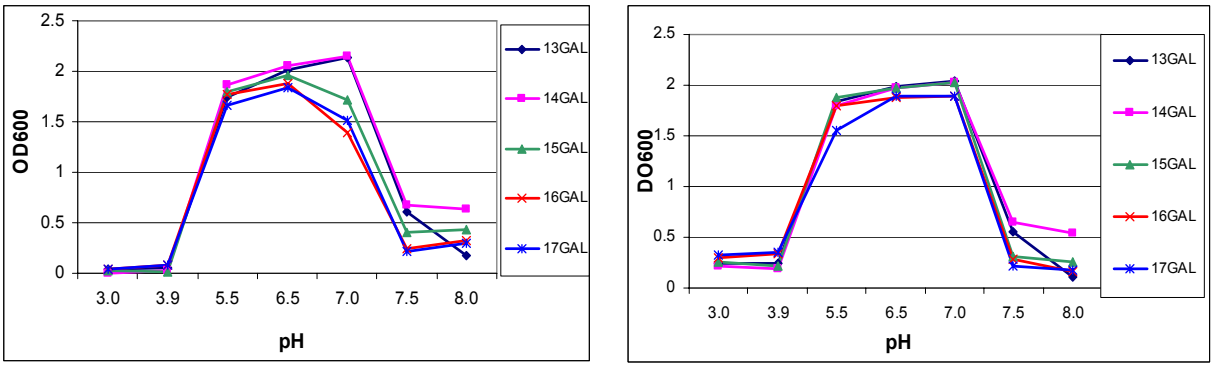

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Figure 5. Growth of the strains at different *p*H values (OD_{600}) at 12h (a) and 24h (b)

From the auxograme it results that the analyzed strains have the ability to metabolize a large number of carbohydrates except xilose and ribose. Arabinose is metabolized only by the strains 13, 14 and 15 (tab. 1).

Table 1. Growth of *Lactobacillus* strains on mediums with different sources of carbon

Strain	Lactose	Glucose	Mannose	Galactose	Maltose	Zaharose	Fructose	Rafinose	Dextrine	Arabinose	Xilose	Ribose
13GAL	+	+	+	+	+	+	+	+	+	+	_	_
14GAL	+	+	+	+	+	+	+	+	+	+	_	_
15GAL	+	+	+	+	+	+	+	+	+	+	_	_
16GAL	+	+	+	+	+	+	+	+	+	_	_	_
17GAL	+	+	+	+	+	+	+	+	+	_	_	_

The strains of the *Lactobacillus* analyzed are catalase-negative, do not metabolize starch, do not reduce tetrazolium salts, do not produce H_2S, grow in the presence of billiary salts, hydrolyze esculine and produce oxygenated water. 13, 14, 15GAL strains are homofermentative, do not hydrolyze arginine and produce flavour substances (acetoine or diacetil) - Voges Proskauer positive reaction. 16, 17 GAL strains are heterofermentative, hydrolyze arginine and they have Voges Proskauer negative reaction. The strains 13 and 16 do not produce polysaccharides (tab.2).

Table 2. Growth of *Lactobacillus* strains on mediums imposed by the methods of the identification

Strain	Production of CO_2	Catalase	Culture at 15°C	Culture at 45°C	Hydrolysis of starch	Production of polysacchar.	Hydrolysis of arginine	Hydrolysis of esculine	Production of H_2O_2	Production of H_2S	R. Voges Proskauer	Reduction of TTC	Resistance to billiary salts
13GAL	-	-	±	+	-	-	-	+	+	-	+	-	+
14GAL	-	-	±	+	-	+	-	+	+	-	+	-	+
15GAL	-	-	±	+	-	+	-	+	+	-	+	-	+
16GAL	+	-	±	+	-	-	+	+	+	-	-	-	+
17GAL	+	-	±	+	-	+	+	+	+	-	-	-	+

Data was processed by means of the Statistica software where references of suspected species were introduced and a graphic resulted (fig. 6) expressing the degree of relationship concerning the strains surveyed with the closest species of *Lactobacillus* (expressed in linkage percentage). The higher the vertical lines, the furthest the species are. Thus: the 13 – 15 GAL isolates are the closest to *Lb. plantarum 1A*, and the 16 – 17 GAL isolates can be either *Lb. brevis,* either *Lb. delbrueckii* or *Lb. plantarum*, too.

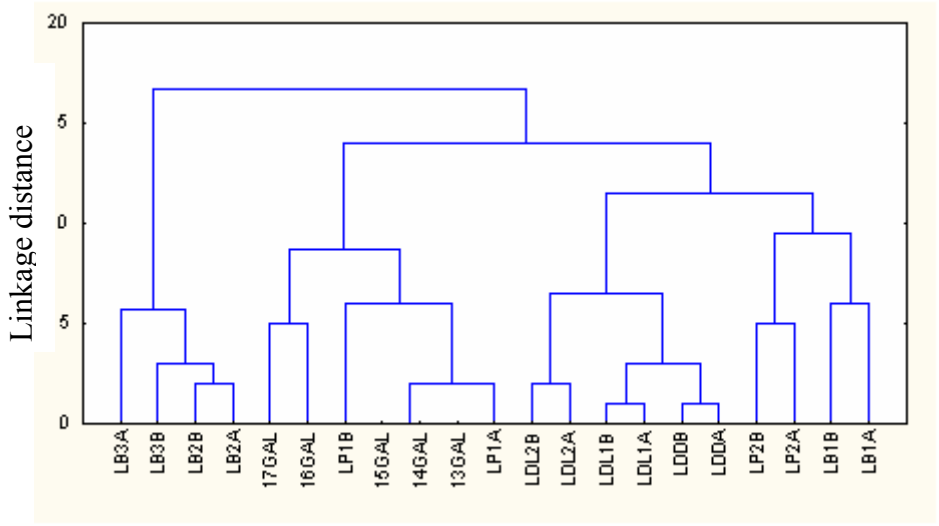

Figure 6. Result of phenotypical analysis applied to 13 – 17 GAL isolates by means of the Statistic software

3. Identification of the lactic acid bacteria strains

The API 50 CHL tests proved that 13GAL, 14GAL, 15GAL strains are *Lactobacillus plantarum* (100% probability). According with the API 50CHL tests, 16GAL, 17GAL strains are *Lactobacillus brevis 1* (96% probability).

The 13 – 17 GAL strains were genetically analyzed using highly sensitive techniques as *in situ* PCR that detect ARN_r 16S and RT - PCR (reverstranscription PCR amplification) that detects the ARN_m particular to each species by means of reverstranscription. The results of these analyses are: 13GAL – *Lb. plantarum*, 14GAL – *Enterococcus sp*. 15GAL – *Lb. plantarum*, 16GAL – unidentified, 17GAL – *Lb. brevis*. These molecular techniques (made in Ghent - Belgium) have not been personally worked and the results were used only for comparison reasons with our results. For the 13GAL, 15GAL, 17GAL strains, results are the same with those achieved by us. The 16GAL strain unidentified by genetic tests was determined as being *Lb. brevis* with a probability of 96%. For the 14GAL strain whose genetic determination is not in line with the results of phenotypical analysis techniques or identification with API 50 CHL tests carried out by us, we tested it in parallel with the API 20 Strep test (BioMerieux).

The numerical profile of the 14GAL strain is **5676771**. The number concerned cannot be identified with the API 20 Strep test software. Therefore the 14GAL strain is not of *Enterococcus* type. Systematic compliance of the 14GAL strain with the *Lactobacillus plantarum* species carried out with the API 50 CHL test is correct.

4. Lactic acid bacteria strains preservation and stability

It is very important that by means of conservation, isolated strains do not modify biotechnological properties and show genetic stability. The selected conservation method should reduce the risk of occurring genetic modifications (spontaneous mutations or plasmids

losses). The preserved cultures should maintain their purity and the selected method should avoid the risk of contamination.

4.1. Preservation by refrigeration

An alternative for a short-time was culture refrigeration at the temperature of 4°C, but they should be often repitching at a time of 7 - 10 - max. 14 days and culture stability is not guaranteed. The extension of repitching period to a month, for conservation at 4°C, was carried out adding 1%$CaCO_3$ in an equimolecular ratio with glucose in the medium and taking into consideration that in a mole of glucose, two moles of lactic acid result by fermentation that should be neutralized with two moles of $CaCO_3$. The viability is guaranteed 2 years.

4.2. Determination of viability of *Lactobacillus* sp. strains during freezing at -70°C

Lactic acid bacteria are extremely sensitive to the metabolite produced (lactic acid) and therefore it was preserved by freezing at -70°C on glass pearls in a protecting medium containing glycerol. The ability of survival is correlated with the age of culture and therefore it is preferred an *over night* culture of 16 to 24 h, at 37°C. The culture was centrifuged for 10 min, at 10000 rpm, and biomass resuspended in 10 ml sterile normal saline solution, and then, centrifugation was repeated. After removing the supernatant, the experiment was repeated in 5ml MRS with glycerol 20% (v/v). Every 1 ml is distributed in 5 cryotubs for each strain and is stored at -70°C [14].

The percentage of cells witch survive at freezing (-70°C), depends on the initial number of cells in culture, hence of their density. The regeneration time from latency status is 2 -3 days.

The *Lb. plantarum* 13GAL and 15GAL strains have a very good viability. They loose less than 10% of the initial number of cells in the first 6 months of freezing at - 70°C (fig.7).

The *Lb. plantarum* 14GAL and *Lb. brevis* 16GAL strains have the lowest survival rate because in the first 24 hours of freezing at -70°C, they loose approximately 50% of the initial number of cells, and then decline is slow (fig.7).

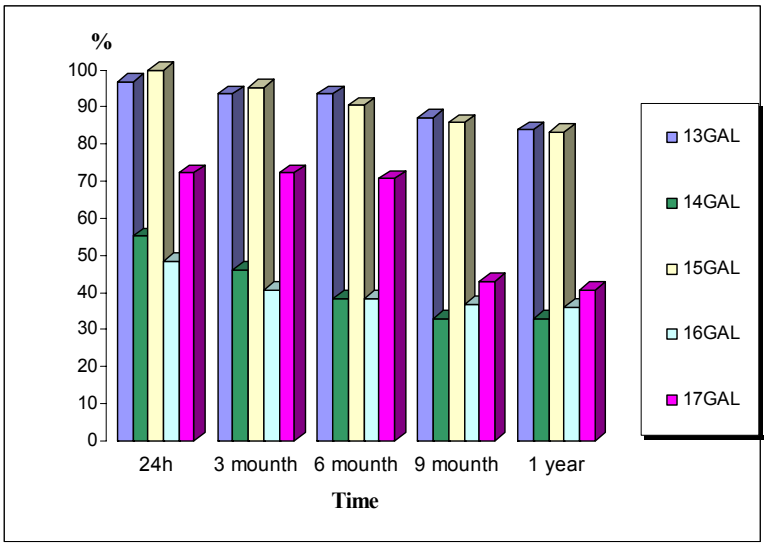

Figure 7. Viability of starter cultures preserved by freezing at -70°C (%)

The *Lb. brevis* 17GAL strain has an intermediary viability between the two former categories because it looses around 30% of the initial number of cells in the first day of freezing, and then viability remains steady up to 9 months, when it experiences a second regression threshold. The most drastic effect of freezing at -70°C is felt over the *Lb. plantarum* 14GAL, *Lb. brevis* 16GAL, 17GAL strains in the first day of freezing.

5. Biotechnological characterization of isolated lactic acid bacteria

The growth dynamics of strains was analyzed based on the CFU/ml criteria and it was noticed the lag phase was the shortest in case of the *Lactobacillus brevis* 16GAL strain, followed by *Lactobacillus plantarum* 14GAL, 15GAL, *Lactobacillus brevis* 17GAL, *Lactobacillus plantarum* 13GAL (fig. 8).

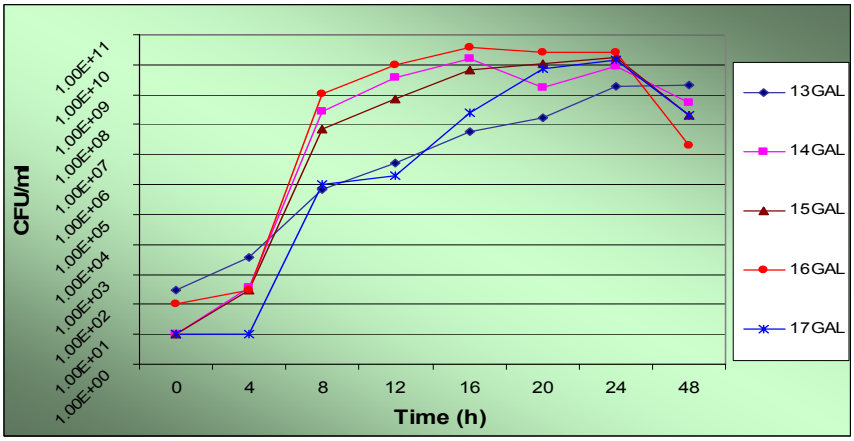

Figure 8. Development of population density concerning selected lactic acid bacteria, during growth in a batch system

The *p*H values is reduced most quickly below 5.5 (after a 12 h - incubation) in case of the *Lactobacillus brevis* 16GAL strain, followed by *Lactobacillus plantarum* 14GAL, 15GAL, *Lactobacillus brevis* 17GAL, *Lactobacillus plantarum* 13GAL (fig.9)

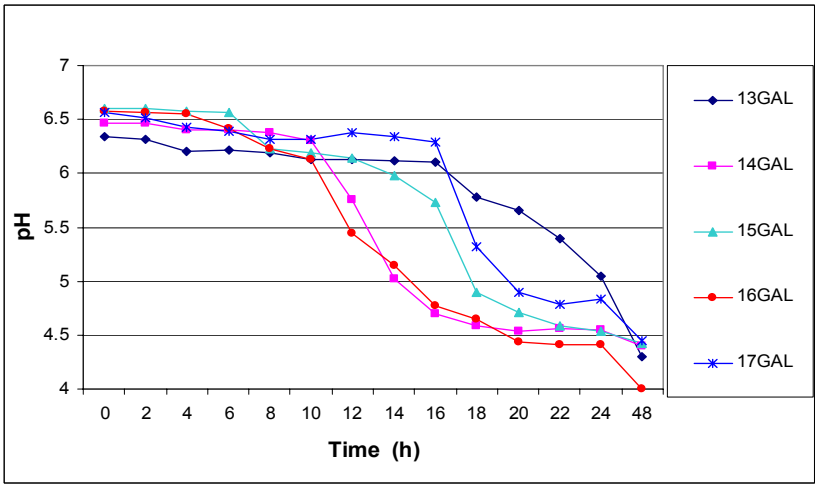

Figure 9. Development of *p*H during growth of *Lactobacillus* selected strains in a batch system

Acidity increases over 5% H_2SO_4 the highest for the *Lactobacillus plantarum* 14GAL strain, followed by *Lactobacillus brevis* 16GAL that reaches the highest acidity (over 7% after an 18 – 20h-cultivation) (fig.10).

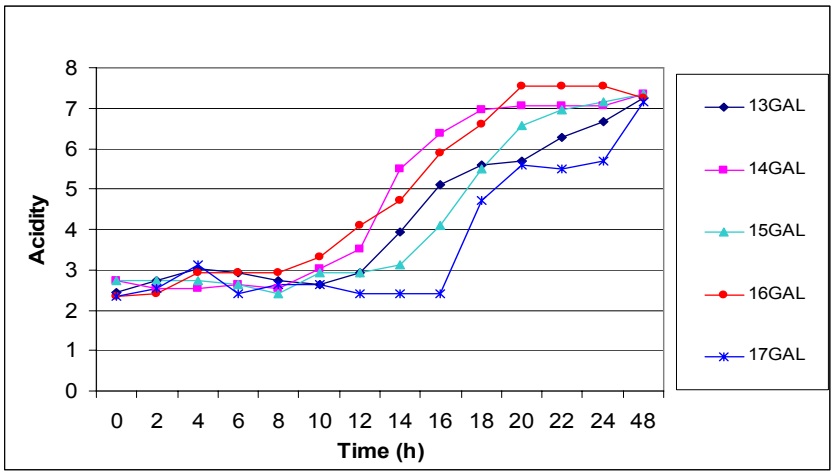

Figure 10. Development of acidity during growth of selected lactic acid bacteria in a discontinuous submerged system

The least biotechnologically favorable strain of the 5 surveyed strains is *Lactobacillus plantarum* 13GAL that only after 22-h cultivation reaches a concentration of $10^8 – 10^9$ CFU/ml. The drop of *p*H and rise of acidity also took place late and slowly.
Following the study of culture behavior in a batch system it is obvious that *Lactobacillus brevis* 16GAL strain has the most suitable growth dynamics for the biotechnological process where it is to be used. The lag phase is short, multiplies quickly, *p*H goes below 5.5 after a 12-h cultivation, acidity increases over 5.5% after a 16 h incubation. Thus, the culture can be used for the surface treatment of germinated wheat after over night cultivation as it has all biotechnological qualities.

Conclusions

1. Five strains of *Lactobacillus* sp. have been isolated from wheat epiphyte microbiota and were identified as *Lactobacillus plantarum* and *Lactobacillus brevis 1*.
2. *Lactobacillus* sp. studied strains grow in a temperature interval of 28°C to 45°C but it develops faster at 45°C than at 28°C. They not prefer salinity higher than 2 – 4% NaCl in medium. The domain of *p*H in which the isolated strains grow is 5.5 – 7.0.
3. The analyzed strains have the ability to metabolize a large number of simple carbohydrates except xilose and ribose.
4. The *Lb. brevis* strains are more susceptible to preservation by freezing compared to *Lb. plantarum* strains that have a better viability after freezing.
5. *Lactobacillus brevis* (16GAL) strain has extremely good biotechnological properties (lag phase was the shortest, the *p*H decreases and acidity increases most quickly after 12 h incubation).

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