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NEW APPROACH FOR FAST SCREENING OF LACTIC ACID BACTERIA FOR VEGETABLE FERMENTATION

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ABSTRACT

The main goal of this research was to select a *Lactobacillus plantarum* strains which could be used as starters for fermented vegetables production. A total of 109 *L. plantarum* strains were screened based on their ability to drop the pH value in pasteurized vegetable-based media (without and with NaCl) after 8 h incubation. The effect of NaCl on the level of acidification after 8 h incubation was more dependent not on its concentration but on the vegetable-based medium and *L. plantarum* strain. Four *L. plantarum* strains were selected and used for lab-scale fermentation of cabbage and cucumbers, this resulted in an accelerated acidification of the vegetables in comparison with the spontaneous fermentation. Strong positive correlations between the differences of initial pH value and the values reached after fermentation during 8 h (ΔpH) on vegetable-based media and vegetable fermentations, suggests that the screening process in vegetable-based media after 8 h incubation could be effective. Four *L. plantarum* strains, selected in this study, can be used as starters for fermented vegetables.

Keywords: fermentation, vegetables, *Lactobacillus*, biotechnology, fermented foods

INTRODUCTION

Fermented vegetables have been produced worldwide for thousands of years mainly for preservation purposes **(Gorny *et al.*, 2006; Das *et al.*, 2016)**. Some traditional methods are still used to manufacture naturally fermented vegetables from raw vegetables. A rapid decrease in pH at the beginning of fermentation is of great importance for the quality of the end product **(Viander *et al.*, 2003)**. But spontaneous fermentation generally needs 4 to 6 days before the pH stabilizes, and depends on the competitive activities of the indigenous microbiota, mainly lactic acid bacteria (LAB). *Lactobacillus plantarum* is one of the species of LAB most frequently found in the spontaneous fermentation of different vegetables, such as cucumbers **(Tamminen *et al.*, 2004)**, table olives **(Harris, 1998)**, kimchi **(Kim and Chun, 2005)** and cabbages **(Karovičová and Kohajdová, 2003; Soltan Dallal *et al.*, 2017)**. The frequent appearance of *L. plantarum* strains during vegetable fermentations makes this species a promising candidate for starter culture trials. A large number of LAB starters are routinely used in dairy and meat industry, but only a few cultures designed for vegetable fermentations **(Montet *et al.*, 2006)**. There are some differences between processes in producing dairy products and fermented vegetables production. The main difference is the inability to inactivate the endogenous microbiota of raw vegetables by a heat treatment without causing drastic changes in their texture. Nevertheless, an increasing number of studies concerning the use of starter culture for fermented vegetables production is published **(Lee *et al.*, 2018; Joyce *et al.*, 2018)**. The most common approach for screening of LAB strains for vegetable fermentation is the evaluation of ability of growth and acidification in sterilized vegetable juices **(Gardner *et al.*, 2001; Di Cagno *et al.*, 2008)**. But the unified evaluation criteria have not been developed. At the same time, there is a criterion for fast evaluation of acidification ability for dairy starters according to the pH value reached in sterile, reconstituted (10% w/v) skimmed milk after 6-8 h incubation **(Cogan *et al.*, 1997; Roushdy, 1999)**. In our opinion, the pH value of pasteurized vegetable-based medium after 8 h incubation might be a good criterion for selection of LAB strains suitable for producing fermented vegetables.

Fermented vegetables have played a significant role in the diet of the population of Ukraine for many years. However, at the moment, there is a lack of commercial bacterial cultures suitable for fermentation of vegetables in Ukraine. Moreover, studies on screening and characterization of *Lactobacillus plantarum* strains with potential use as starter cultures for vegetables fermentation in Ukraine are limited. In our previous works a total of 109 strains of *L. plantarum* were isolated from Ukrainian dairy products and fermented vegetables and these were studied for various biochemical and functional properties **(Vasyliuk *et al.*, 2014a, b)**. So, starter cultures with desirable properties are of particular importance for the production of fermented vegetable products with functional properties (**Soltan Dallal *et al.*, 2017; Behera *et al.*, 2018**).

Hence, the aim of this work was to assess the potential of *L. plantarum* strains, isolated from Ukrainian fermented products, for fermented vegetable production. For this purpose, the suitability of a simple approach, based on the pH value of pasteurized vegetable-based media after 8 h incubation, as a criterion for possible vegetable starter cultures, was assessed. Selected *L. plantarum* strains were successfully applied on lab-scale for fermentation of cabbage and cucumbers, following a traditional production method.

MATERIAL AND METHODS

Bacterial strains

One hundred and nine *L. plantarum* strains isolated from various homemade fermented foods and sampled in different regions in Ukraine were used **(Vasyliuk *et al.*, 2014)**. All strains were maintained in MRS broth, with the addition of 30% (w/v) glycerol at -50 °C in the LAB Culture Collection of the Department of Physiology of Industrial Microorganisms, Zabolotny Institute of Microbiology and Virology NAS, Kiev, Ukraine. The cultures were activated by two successive transfers in the MRS broth before use.

Initial screening

For the initial screening of the strains, a vegetable-based media were produced. Fresh cabbage and cucumbers were washed, cut and separately homogenized in sterile distilled water, at ratios of 1:1 (vegetable : water). NaCl was added at the concentration of 2.5% (w/v) to cabbage-based medium and 6.0% to cucumber-based medium. Media without NaCl are also used. All media were pasteurized at 80 °C for 20 min, cooled and immediately inoculated with 1% (v/v) of an overnight culture of tested strains. Media were incubated for 8 h at 30 °C and acidification was determined by measurement of the pH of samples and expressed as the difference in pH between the initial pH value and the value reached after 8 h (ΔpH). Non-inoculated media were used as a control (Table 1).

Table 1 The pH values of vegetable-based media

Medium	initial pH value		pH value after 8 h (fermentation at 30° C)	
	non-pasterized	pasterized	non-pasterized	pasterized
Cabbage-based medium	5.66±0.31	5.68±0.28	5.41±0.28	5.65±0.32
Cucumber-based medium	5.72±0.22	5.62±0.31	5.46±0.32	5.56±0.30

Vegetables fermentation

Fresh cabbage was shredded, mixed with NaCl (2.5% w/w) and placed in 500 ml glass jars (500 g of cabbage in each jar). Fresh cucumbers were washed and placed in 1 l glass jars and 6.0% NaCl solution (w/v) was added in each jar (520±5 g of cucumbers with 460±5 ml of brine). Jars with salted vegetables were inoculated with 1% (v/v) of an overnight culture of *L. plantarum* strain to achieve an initial level of 10^7 cfu g^{-1}. The vegetables were mixed with inocula and packed tightly. Salted but not inoculated vegetables were used as a control, allowing a spontaneous fermentation. The fermentation was initiated at 30°C for 2 days, after which the jars were stored at 10 °C for the remainder of the fermentation. The fermentation process was performed in duplicate.
Brine and vegetable particles were sampled at specific time points throughout the fermentations, namely at day 0, 1, 2, 3, 14, 21, 28, and after 3 months. All samples were immediately analyzed for acidification and total lactic acid bacteria (LAB) count.

Statistical analysis

A one-way analysis of variance (ANOVA), multiple mean comparisons using LSD test (LSD - little significant difference), cluster analysis and graphing procedures were carried out with the Statistika 7.0 software (Systat Inc., USA). Results from two independent assays were averaged. Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Growth of *L. plantarum* strains in vegetable-based media

At the first stage, 109 *L. plantarum* strains were screened based on the ability to grow in vegetable-based media. After 8 h of fermentation at 30 °C, the values of ΔpH (the difference in pH between the initial pH value and the value reached after 8 h) ranged from 0.125±0.02 to 2.33±0.01, depending on the medium and strain. A hierarchical cluster analysis was performed and grouping of *L. plantarum* strains on the base of ΔpH values was mostly related to the differences of ΔpH values on medium without NaCl and corresponding medium with NaCl and was unrelated with source of the strains (Fig. 1). For 10% strains, NaCl addition did not affect the ΔpH values in both vegetable-based media (clusters I and III), whereas for 33% strains the pH values were lower than in the corresponding media without salt (clusters IV and VII). For other strains, the effect of NaCl on ΔpH values was different depending on the vegetable-based medium. For 42.2% strains in cabbage-based medium with 2.5% NaCl the ΔpH values were lower, but in cucumber-based medium with 6% NaCl the ΔpH values were the same, compared with corresponding media without salt (clusters II and VI). For 11% strains (cluster VIII), the ΔpH values for at least one of the vegetable-based media with salt (mainly cucumber-based with 6% NaCl) were higher in comparison with the corresponding medium without salt, whereas in the other medium the ΔpH values did not change or were lower. So, an interesting observation made during this study was that the effect of NaCl on the level of acidification after 8 h incubation was more dependent not on its concentration but on the vegetable-based medium and *L. plantarum* strain. For many *L. plantarum* strains in cabbage-based medium with low NaCl concentration (2.5%) the ΔpH values were lower but in cucumber-based medium with high NaCl concentration (6.0%), the ΔpH values were higher, compared to corresponding NaCl-free media.

Vegetable fermentation

Starter cultures with desirable functional properties, apart from the technological aspect, are of particular interest in terms of producing fermented vegetables with improved quality and usefulness for people health **(Tamang *et al*., 2009; Rezac *et al*., 2018; Behera *et al*., 2018)**. Thus, in addition to acidification capacity in vegetable-based media, we took into account the functional properties of *L. plantarum* strains **(Vasyliuk *et al*., 2014a,b; Livinska *et al*., 2016)**. So, four *L. plantarum* strains, namely *L. plantarum* 47SM (isolated from soured cream), 1047K, 952K (isolated from fermented cabbage) and 691T (isolated from cottage cheese) were selected to study their suitability as starter cultures for fermented cabbage and cucumbers.
The pH of the inoculated by *L. plantarum* strains cabbage was significantly (P<0.05) lower than the control (Fig. 2a), whereas there were no significant differences in pH decreases between spontaneous fermented and inoculated cucumbers after 24 h (Fig. 2c). After a 48 h fermentation process, the pH values of inoculated cabbage samples decreased to a level below than 3.4. At the same time, the values of pH for cucumbers fermented with *L. plantarum* strains were in the range from 3.56-3.74. After 48 h incubation, the values of pH of spontaneously fermented vegetables were higher than those with *L. plantarum* strains.
The LAB growth dynamics significantly differed during fermentation of cabbage and cucumbers similarly to the difference in medium acidification dynamics. The counts of presumptive LAB were significantly higher after 24 h of incubation in the inoculated cabbage in contrast to control cabbage fermented spontaneously (Fig. 2b). However, there was no difference (P>0.05) in presumptive LAB counts between control and *L. plantarum*-inoculated cabbage after 48 h. After 24 h fermentation, LAB cell count was not differed between control and inoculated cucumbers. The increase of presumptive LAB in cucumbers inoculated with *L. plantarum* 47SM occurred after 48 h becoming significantly higher compared to spontaneously fermented cucumbers (Fig. 2d). Overall, there were no significant differences in presumptive LAB counts and pH values between spontaneous fermented and inoculated vegetables under refrigeration storage during 3 months.

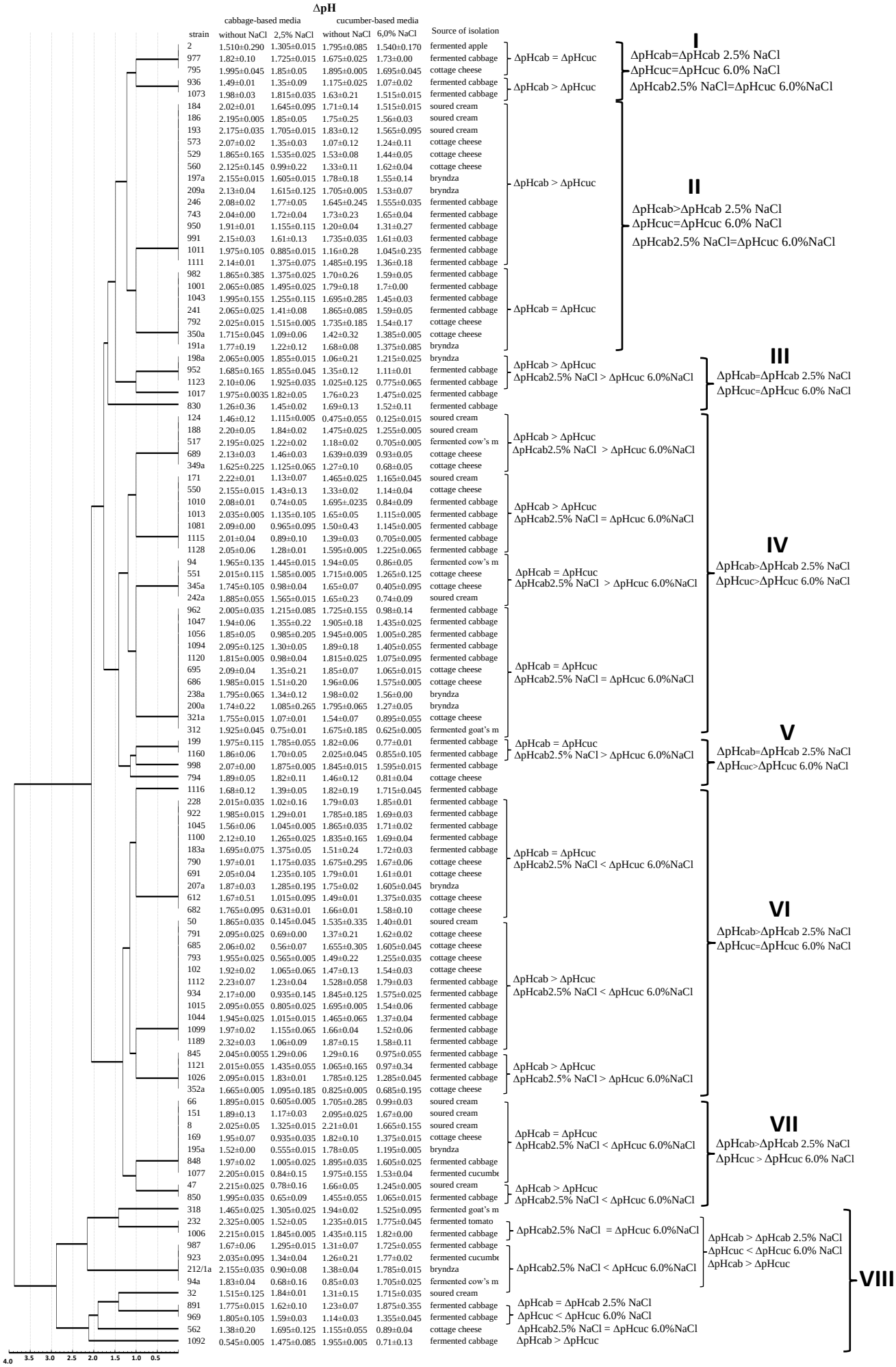

Figure 1 Dendrogram based on differences between ΔpH values obtained after 8 h incubation of *L. plantarum* strains in different vegetable-based media: ΔpHcab – cabbage-based medium; ΔpHcab 2.5% NaCl - cabbage-based medium with 2.5% NaCl; ΔpHcuc – cucumber-based medium; ΔpHcab 6.0% NaCl - cucumber-based medium with 6.0% NaCl; A cluster analysis was conducted with similarity estimates by using UPGMA.

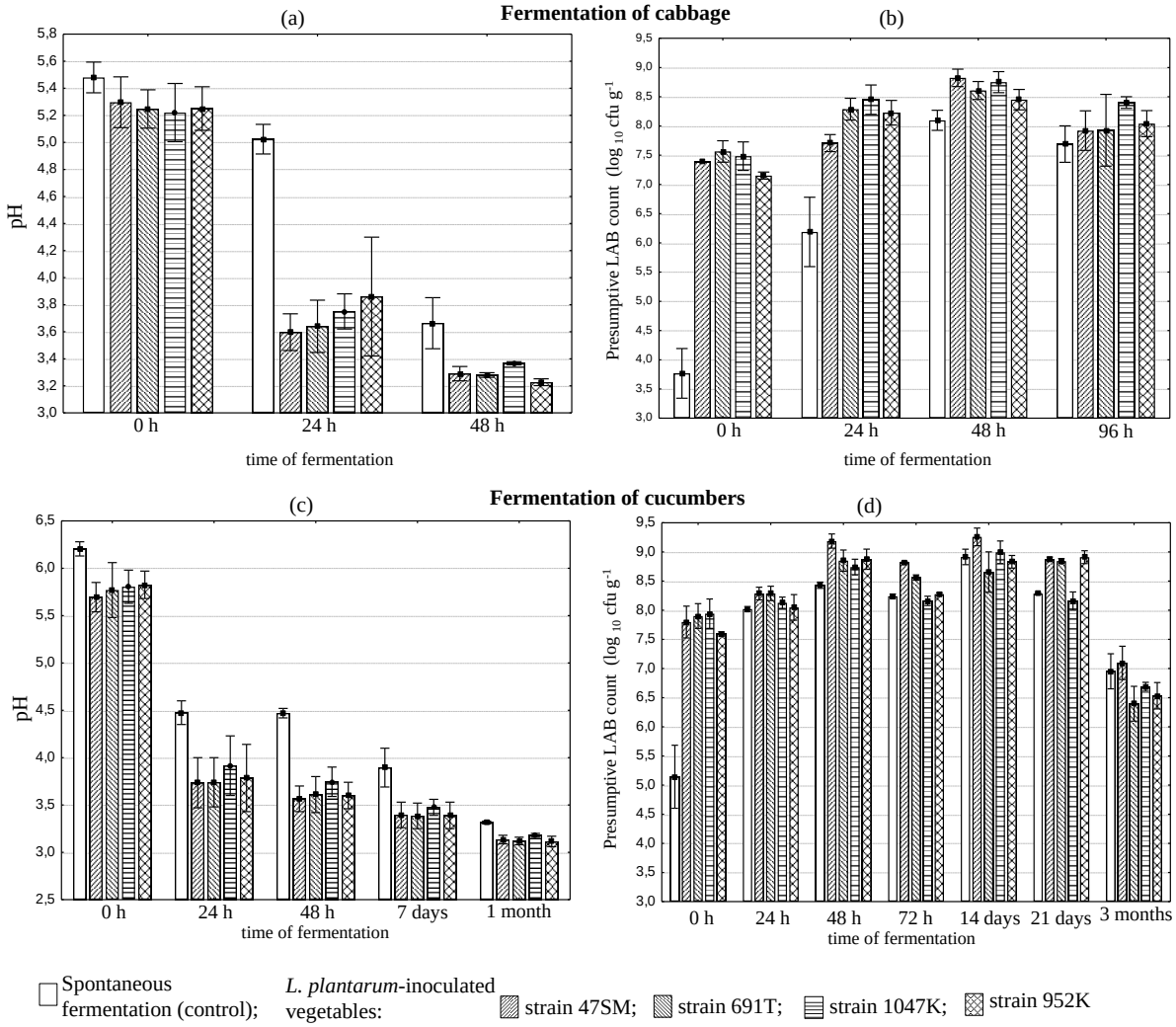

Figure 2 Changes in pH and presumptive lactic acid bacteria (LAB) counts during fermentation of vegetables

Four *L. plantarum* strains, selected for vegetable fermentation, had a different ΔpH values on pasteurized vegetable-based media. But there was no statistically significant difference between the vegetables inoculated by different *L. plantarum* strains. (Table 2).

Table 2 The ΔpH values on vegetable-based media and vegetables inoculated by *L. plantarum* srains

Strains	Media/vegetables, time of fermentation, ΔpH							
	cabbage-based medium, 8 h		cabbage+2.5% NaCl		cucumber-based medium, 8 h		cucumbers+6% NaCl	
	pasteurized	pasteurized + 2.5% NaCl	24 h	48h	pasteurized	pasteurized + 6.0% NaCl	24 h	48h
Control (non-inoculated)	0.03±0.02	0.05±0.01	0.45±0.07	1.81±0.08	0.06±0.05	0.04±0.04	1.73±0.28	1.73±0.03
L. plantarum 47SM	2.21±0.03	0.78±0.22	1.70±0.05	2.00±0.13	1.66±0.07	1.24±0.01	1.96±0.15	2.13±0.02
L. plantarum 691T	2.05±0.05	1.23±0.14	1.60±0.29	1.96±0.14	1.79±0.01	1.61±0.01	2.03±0.04	2.16±0.14
L. plantarum 1047K	1.94±0.08	1.35±0.30	1.47±0.13	1.84±0.18	1.90±0.24	1.43±0.03	1.89±0.19	2.00±0.02
L. plantarum 952K	1.68±0.23	1.85±0.06	1.39±0.29	2.02±0.13	1.35±0.16	1.11±0.01	2.04±0.29	2.22±0.01

Multivariate analysis for the correlation between values of ΔpH was performed of the four *L. plantarum* strains depending on vegetable-based media and vegetables (Table 3). There were significant strong positive correlations between values of ΔpH, that were found on vegetable-based media and values of ΔpH on fermented vegetables.

The selection of LAB as suitable starters for production of fermented vegetables is a multi-stage process. The most common approach is the use of sterilized vegetable-based media, mainly vegetable juices that can be stored at −20 °C before use **(Gardner *et al*., 2001; Di Cagno *et al*., 2008)**. The kinetics of growth and acidification were determined by authors after incubation during 24 h **(Di Cagno *et al*., 2008)** or up to 60 h **(Gardner *et al*., 2001)**. As was shown by **Di Cagno et al. (2008)**, acidification for the lactic acid bacteria strains has reached values of ΔpH which ranged from 2.32 to 3.05 after 24 h of incubation in vegetable juice media. In the present study, after 8 h of incubation, the pH values of inoculated vegetable-based media decreased by 0.125- 2.33 units from initial value, whereas in control media – only on 0.03-0.06 units. The decrease of pH reflects the growth of lactic acid bacteria and these results indicate that the *L. plantarum* strains studied were able to proliferate in the vegetables-based media used. So, the screening approach used in this work is simple and less time consuming as compared to other screening methods.

The most studies on LAB resistance to NaCl have been performed using MRS medium, the standard rich medium used for laboratory cultivations **(Bevilacqua *et al*., 2010)**. But the cultivation of *L. plantarum* on MRS has to be regarded as significantly different from the conditions encountered by *L. plantarum* during vegetable fermentations, as was shown by authors **(Di Cagno *et al*., 2013)**. As was shown by **Filannino *et al.* (2016)**, the most prominent phenotypic dissimilarities observed in cells grown on carrot and pineapple were related to carbon and nitrogen metabolism, respectively. It's well known that the bacterial response to hyperosmolarity is related to the ability of cells to accumulate compatible solutes that include amino acids, amino acid derivatives, polyols, and sugars **(Csonka, 1989)**. To our knowledge, no papers in which the effect of different concentration of NaCl on growth *L. plantarum* strains in several vegetable-based media has been compared were published, and this makes it very difficult to compare results of our work with those obtained by others authors. Further studies are required to determine the mechanisms responsible for the NaCl resistance during growth in vegetables.

Table 3 Correlations between ΔpH values on vegetable-based media and vegetables inoculated by *L. plantarum* srains

Media/vegetables, time of fermentation	Vegetables-based media, 8 h incubation				Fermented vegetables			
	cabbage-based media		cucumber-based media		cabbage (2.5% NaCl)		cucumbers (6% NaCl)	
	without NaCl	2.5% NaCl	without NaCl	6.0% NaCl	24 h	48 h	24 h	48 h
Vegetables-based media, 8 h								
cabbage-based	1.0							
cabbage-based+2.5% NaCl	0.67	1.00						
cucumber-based	**0.96***	0.69	1.00					
cucumber-based +6.0% NaCl	**0.95***	0.73	**0.98***	1.00				
Fermented vegetables								
cabbage (2.5% NaCl), 24 h	**1.00***	0.67	**0.95***	**0.95***	1.00			
cabbage (2.5% NaCl), 48 h	0.64	0.56	0.44	0.51	0.68	1.00		
cucumbers (6.0% NaCl), 24 h	0.82	0.83	0.73	0.81	0.84	0.87	1.00	
Cucumbers (6.0% NaCl), 48 h	**0.88***	0.88	0.82	0.86	**0.90***	0.83	**0.98***	1.00

Dynamics of pH decrease and presumptive LAB count increase during fermentation of inoculated cabbage were different from those of cucumbers. This may be due to a smaller number of the indigenous presumptive LAB in raw cabbage than in cucumbers (3.76±0.73 $\log_{10}$ cfu g^{-1} and 5.14±1.08 $\log_{10}$ cfu g^{-1}, respectively). And although, *L. plantarum* strains addition in high numbers ensured a prevalence of these strains in the inoculated vegetables, the inoculated *L. plantarum* strains competed not only with indigenous LAB, but with all microorganisms of the raw vegetables. Many authors have reported that the use of lactic acid bacteria strains as starters in the fermentation of vegetables significantly increased acid production, when compared with spontaneously fermented vegetables **(Desai and Sheth, 1997; Joyce *et al*., 2018)**.

Difference in lactic acid production rate by *L. plantarum* strains during growth on vegetable-based media and vegetables fermentation may be due to the fact that vegetable-based media were pasteurized, in contrast to raw vegetables with native microflora. Another reason may be the difference in the nutritional composition of cabbage and cucumbers and their availability. So, for the preparation of vegetable-based media the vegetables were homogenized. Before fermentation, the cabbage was shredded, while cucumbers were fermented whole. The significant strong positive correlations between ΔpH values on cabbage- and cucumber-based media and significant strong positive correlations between ΔpH values on vegetable-based media and fermented cabbage after 24h incubation may also indicate an increase in the availability of nutrients. At the same time, significant correlations between cucumber-based media and fermented cucumbers were not detected.

CONCLUSION

In conclusion, to our knowledge, this is a first study in which a simple and fast approach was used to evaluate the potential of *L. plantarum* strains as starters for vegetable fermentation. Stimulating effect of NaCl in vegetable-based medium on the acidification, during exponential growth phase of *L. plantarum* strains has been found for the first time. The four *L. plantarum* strains selected through this research are promising starter cultures, as was shown in the laboratory-scale fermentation of vegetables. The impact of these strains on the organoleptic properties of the fermented vegetables will be the object of further investigation. Strong positive correlations were found between values of ΔpH on vegetable-based media (without NaCl) and values of ΔpH that were found during vegetable fermentations, which suggests that the screening process in vegetable-based media after 8 h incubation could be effective.

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