ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 12 (5): 202-208, 2013
ISSN 1682-296X / DOI: 10.3923/biotech.2013.202.208

Evaluation of *Lactobacillus reuteri* Strains for Pumpkin (*Cucurbita pepo* L.) Juice Fermentation

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Abstract: Recently demand for non-dairy-based probiotic products has increased and is a global trend. Recently great interest is arising for vegetable and especially vegetable juices fermentation for development of non-dairy functional products. Health promoting properties of pumpkin (*Cucurbita pepo*) is well known but there are only few data on pumpkin juice fermentation. Starter cultures have great influence on product organoleptic properties and therefore careful selection of cultures suitable for fermentation of certain raw materials is required. Recent studies marked out probiotic strains of *Lactobacillus reuteri* between other probiotics but there are no data on application of *L. reuteri* strains for juice e.g., pumpkin fermentation. The objective of this study was to determine the suitability of *L. reuteri* strains for fermentation of pumpkin juice and development of fermented potentially probiotic pumpkin juice-based beverage. *L. reuteri* strains used in the study grew well in pumpkin juice however strain specific growth trends were observed. Salt tolerance combined with very good sensory properties was chosen as characteristic parameters to select most prospective *L. reuteri* strains. It was shown that pumpkin juice supplementation with additional sugars (glucose, fructose or sucrose 1-4%) did not promote fermentation process and was disadvantage. *L. reuteri* strains exhibited excellent survival during 4 weeks cold storage of fermented pumpkin juice at the level of 10^9 CFU mL^{-1} that is sufficient for probiotics effective supply with food. It was concluded that application of certain *L. reuteri* strains for pumpkin juice fermentation is a promising way for development of novel non-dairy based probiotic fermented beverage.

Key words: Pumpkin, fermented juice, *Lactobacillus reuteri*, probiotic, beverage

INTRODUCTION

In recent years consumer demand for non-dairy-based probiotic products has increased and is a global trend. It is related to increasing numbers of individuals that are lactose intolerant and/or have milk protein allergy. Thus plant origin food could be valuable alternative and new potential carriers for probiotics should be evaluated. In addition vegetarianism and veganism are becoming popular in many countries.

The nutritional potential of vegetables is remarkable-they are rich in minerals, vitamins, dietary fibers and antioxidants. Preservation of foods by lactic acid fermentation is widely practiced technology since elderly. Fermentation improves digestibility of vegetables and positively influences availability of some minerals and vitamins (Steinkraus, 1996; Buckenhuskes, 1997). Fermented foods have better flavour/aroma and improved health effects and safety. Thus fermentation allows improving food preservation in natural way and upgrades substrates to higher value products. The most common and well-known fermented vegetables are cabbage, cucumbers, olives as well as regionally important cultures throughout the world. Recently great interest is arising for other vegetable and especially vegetable juices fermentation for development of non-dairy functional products (Prado *et al*., 2008). Vegetable juices similarly to vegetables contain beneficial nutrients; besides, due to good taste properties, could be better approved by consumers especially taking into account that generally vegetable juices are perceived as healthy. There are data on tomatoe (Yoon *et al*., 2004; Kohajdova *et al*., 2006), red beet (Yoon *et al*., 2005; Rakin *et al*., 2007), carrot (Kohajdova *et al*., 2006; Kun *et al*., 2008; Tamminen *et al*., 2013), cucumber (Buruleanu *et al*., 2011), pepper (Buruleanu *et al*., 2012) and other vegetable juice fermentation with lactic acid bacteria (LAB) cultures.

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Pumpkin is a well-known low calorie vegetable (100 g fruit provides just 26 kcal) cultivated worldwide in a big scale. Pumpkin is rich in β-carotene, gamma-aminobutyric acid, minerals like copper, calcium, magnesium, potassium and phosphorus and dietary fibers (Yadav *et al*., 2010). With B-complex group of vitamins like folates, niacin, vitamin B-6 (pyridoxine), thiamin and pantothenic acid, vitamin C, E and vitamin A pumpkin is featuring highest levels of vitamins. Pumpkin pulp was used for the production of oligosaccharides that were resistant to artificial human gastric juice and favoured growth of lactobacilli (Du *et al*., 2011). Anti-diabetic, antioxidant and anti-carcinogenic properties are attributed to pumpkin compounds (Caili *et al*., 2006; Kwon *et al*., 2007; Baldi *et al*., 2010).

There are only few data on pumpkin juice fermentation. It was reported that pumpkin juice was fermented by *Lactobacillus plantarum* CCM 7039 starter culture. The culture was able to grow in pumpkin juice, pH dropped down to 3.35 and 15.19 g dm^{-3} of lactic acid was produced (Kohajdova *et al*., 2006). However in sensory evaluation it was concluded that fermented pumpkin juice had rather flat flavour. It is known that besides fermenting substrate starter cultures have great influence on product organoleptic properties (De Vuyst, 2000) and therefore careful selection of cultures suitable for fermentation of certain raw materials is required.

Fermentation rate and production of total acidity, rapid pH decrease, reduction of concentrations of nitrates and nitrites, reduction of biogenic amines and beneficial influence of organoleptic properties of the product are the characteristic features required for industrial starter cultures (De Vuyst, 2000). Mainly LAB starter cultures are used for industrial scale vegetable and vegetable juices fermentation. However fermentation of non-dairy substrates by LAB cultures, especially probiotic, is still a challenge for both scientists and an industry due to several reasons: poor growth in fermenting substrate or fermenting substrate not suitable for probiotic starter growth; technological regime not suitable for probiotic growth; probiotic starters do not provide necessary organoleptic properties for product; characteristics of fermented substrate (e.g., acidity, natural inhibitory substances, salt or spices adittion) do not provide sufficient cell count of probiotic bacteria in final product (Mattila-Sandholm *et al*., 2002). Industrially applicable LAB starter cultures are characterized by rapid pH reduction of a substrate due to production of lactic acid and acetic acid, synthesizing several other inhibitory primary metabolites as hydrogen peroxide, carbon dioxide, diacetyl and antimicrobial compounds that can extend shelf life and improve safety of fermented food products (Kohajdova *et al*., 2006).

Recent studies marked out probiotic strains of *L. reuteri* between other probiotics. As it is important for vegetable juices fermentation, growth of *L. reuteri* in this substrate is better as compared with bifidobacteria. *Lactobacillus reuteri* is a resident of the human and animal gastrointestinal tracts (Casas and Dobrogosz, 2000). *L. reuteri* is also considered to be indigenous to the human gastrointestinal tract (Reuter, 2001). Certain strains of *L. reuteri* have been proved to possess health-promoting effects e.g. prevention of lactose maldigestion (Ojetti *et al*., 2010), diarrhoea (Wolf *et al*., 1995; Shornikova *et al*., 1997) and hypercholesterolaemia (Jones *et al*., 2012). Besides *L. reuteri* produce reuterin (3-hydroxypropionaldehyde) that is broad-spectrum antibiotic (Talarico *et al*., 1988; Talarico and Dobrogosz, 1989; Doleyres *et al*., 2005). During recent years, *L. reuteri* has been widely used as a probiotic supplement in dairy-based functional foods (Casas and Dobrogosz, 2000; Hernandez-Mendoza *et al*., 2007).

The objective of this study was to determine the suitability of *L. reuteri* strains for fermentation of pumpkin juice and development of fermented potentially probiotic pumpkin juice-based beverage.

MATERIALS AND METHODS

Strains: The strains of *Lactobacillus reuteri* used in the study were obtained from the Collection of Microorganisms of the Institute of Microbiology and Biotechnology, University of Latvia.

Media and growth conditions: MRS growth medium (De Man *et al*., 1960) was used for the maintenance and propagation of the cultures.

Pumpkin (*Cucurbita pepo*) were from a Latvian local market and juice was obtained using a mechanical squeezer. Fresh pumpkin juice was pasteurized at 60°C for 20 min. Pumpkin juice contained 4.8% dw, 13.4 g L^{-1} glucose, 15.8 g L^{-1} fructose and 8.9 g L^{-1} sucrose. The initial pH was 6.05, titrable acidity 23°T. Bacteria strains were grown at +37°C in closed 250 mL Erlenmeyer flasks for 48 h.

Pumpkin juice was supplemented with sucrose, glucose, fructose and seasonings (dill, garlic, caraway) when appropriate.

Analytical measurements: The growth of *L. reuteri* strains was monitored by optical density (OD) spectrophotometric measurement at 550 nm (Helios Gamma, Thermo Scientific, UK).

Total titratable acidity was determined by alkaline titration (0.1 mol L^{-1} NaOH) of the samples, using phenolphthalein as the indicator and was expressed in Thörner degrees (°T).

The concentrations of organic acids (lactic, acetic, gluconic, succinic and citric) were quantified by HPLC (Agilent 1100, HP, USA) with a refraction array detector, column Shidex SH 1011, column temperature 50°C, mobile phase 0.01 N H_2SO_4 and flow 0.6 mL min^{-1}.

The concentration of ethanol were quantified by HPLC (Agilent 1100, HP, USA) with a refraction detector, column Shidex SH 1011, column temperature 50°C, mobile phase 0.01 N H_2SO_4 and flow 0.6 mL min^{-1}.

The viable cell count was monitored by the spread-plate method using the agarized MRS medium.

To determine bacteria viability during storage, after fermentation the samples were stored in the dark at +4°C for 2 weeks.

The concentration of carbohydrates (glucose, fructose and sucrose) were quantified by HPLC (Agilent 1100, HP, USA) with a spectrophotometric detector (wavelength 210 nm). Data was analyzed using the Agilent Chemistation program. The content of the fructans was determined according to the AOAC methods AOAC-99.03 and AACC32.32.

Sensory evaluation: The overall pleasantness of taste and flavour were assessed using 100 mm graphical non-structured line segments with specified end-points and was expressed as a percentage of the scale.

Statistical analyses: The data presented are from at least three independent cultivations. All analytical measurements were repeated five times. The Student's t-test was employed to check the differences between means at a significance level <0.05.

RESULTS

It is known that salt (NaCl) commonly is used for undesirable microflora growth reduction, organoleptic properties improvement and juice release facilitation in vegetable juice fermentation (Holzapfel *et al*., 2003; Viander *et al*., 2003). Besides NaCl affects also the metabolism and growth of LAB involved in fermentation process (Chikthimmah *et al*., 2001; Johanningsmeier *et al*., 2012). It is reported that the presence of a higher level of NaCl (5%) inhibited the growth of LAB (Chikthimmah *et al*., 2001).

It was shown that addition of NaCl differently influenced the pH and total acidity of pumpkin juice fermented with various *L. reuteri* strains (Fig. 1a, b). The pumpkin juice pH at the end of fermentation was lower in the presence of 0.75% NaCl than without NaCl for strains *L. reuteri* 25, 42, 44, 45 and 16 (Fig. 1a). On contrary, addition of 0.75% NaCl significantly increased (influenced positively) the total acidity of pumpkin juice fermented only with *L. reuteri* 45 (Fig. 1b). For other *L. reuteri* strains were observed opposite effect of NaCl addition on juice total acidity (Fig. 1b).

The addition of NaCl influenced also consumption of glucose and fructose during pumpkin juice fermentation by *L. reuteri* strains (Fig. 1c, d). It was detected that glucose, as well as fructose consumption was better without NaCl, especially for strains *L. reuteri* 45 and 16 (Fig. 1c, d). In addition, the presence of 0.75% NaCl during pumpkin juice fermentation inhibited the acetate and lactate formation by *L. reuteri* 45 and 16 strains (Fig. 1e, f). It was also shown that strains *L. reuteri* 44 and 19 produced acetate in very small amounts that could be positive factor considering final product organoleptic properties (Fig. 1e).

Bearing in mind complete profile of fermentation patterns it was concluded that most prospective strain for pumpkin juice fermentation could be *L. reuteri* 19-the final product had low pH and high titratable acidity, glucose and fructose was considerably consumed and strain produced high amounts of lactate and very low amount of acetate. The most sensitive strain to NaCl addition was *L. reuteri* 16, as illustrated by high pH and low titratable acidity. Subsequently consumption of glucose and fructose in the presence of NaCl was low for strain *L. reuteri* 16.

Tolerance to different NaCl concentrations (0-2%) was evaluated for chosen strains 44, 25 and 16 (Fig. 2a, b). It was shown that assessed strains expressed different salt tolerance patterns. The changes of pH during fermentation by *L. reuteri* 16 were rapid and were not influenced significantly by NaCl concentrations added (Fig. 2a). Although the total acidity after 96 h fermentation by *L. reuteri* 16 was the highest at all NaCl concentrations among evaluated strains (Fig. 2b), the highest total acidity was estimated for control sample without NaCl addition. Yet strain *L. reuteri* 16 was most tolerant to presence of NaCl. For strain *L. reuteri* 25 concentration dependent effects was observed, especially on total acidity. The growth of strain

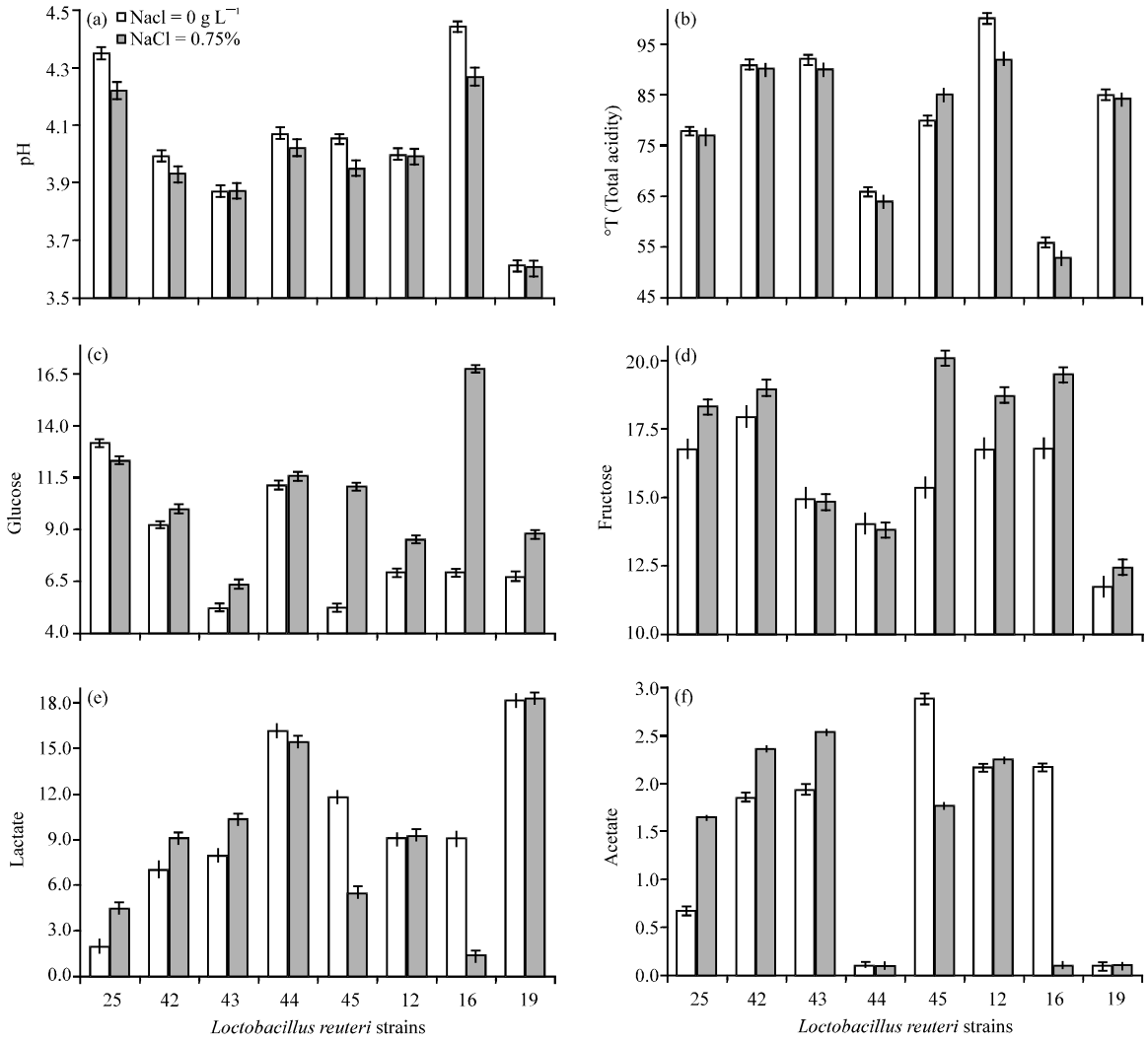

Fig. 1(a-f): (a) pH, (b) Titratable acidity °T, (c) Glucose (g L^{-1}), (d) Fructose (g L^{-1}), (e) Lactate (g L^{-1}) and (f) Acetate (g L^{-1}) concentrations at the end of pumpkin juice fermentation (48 h) with various *L. reuteri* strains

L. reuteri 44 was quite week and no significant NaCl concentration dependent effect was observed, so it can be concluded that strain 44 is not suitable for pumpkin juice fermentation.

It was shown previously that fermented pumpkin juice had quite flat flavour (Kohajdova *et al*., 2006). Therefore different seasonings were added to pumpkin juice before fermentation in order to improve product organoleptic properties.

It was shown that seasonings did not influence the biomass formation of *L. reuteri* 42 (Table 1), however combination of 1 g L^{-1} dill+1 g L^{-1} caraway+1 g L^{-1} garlic significantly enhanced the acidification rate during fermentation.

Table 1: Influence of different seasonings on biomass production and total acidity of pumpkin juice after 48 h fermentation by *L. reuteri* 42

Seasonings	Biomass (g L^{-1})	°T (total acidity)
0.6 g L^{-1} dill+0.6 g L^{-1} caraway	0.80±0.02	61±2
1 g L^{-1} dill+1 g L^{-1} caraway	0.80±0.02	76±2
0.6 g L^{-1} dill+0.6 g L^{-1} caraway +0.6 g L^{-1} garlic	0.80±0.02	86±2
1 g L^{-1} dill+1 g L^{-1} caraway +1 g L^{-1} garlic	0.78±0.02	90±2
0.6 g L^{-1} caraway+ 0.6 g L^{-1} garlic	0.80±0.02	75±2

The viability of probiotic cultures during the whole period of product storage is the most important functional product quality indicator. It was reported that viability of probiotic bacteria depend on the level of oxygen in

Table 2: Viability of (CFU mL^{-1}) *L. reuteri* strains during fermented pumpkin juice storage (+4°C)

Strains	After fermentation	2 weeks	4 weeks	Cell count changes during storage
L. reuteri 25	3.1×10^{9}	3.0×10^{8}	2.8×10^{7}	3.0×10^{9}
L. reuteri 42	2.5×10^{9}	5.0×10^{8}	2.5×10^{7}	2.5×10^{9}
L. reuteri 43	1.2×10^{9}	5.4×10^{8}	2.0×10^{7}	1.2×10^{9}
L. reuteri 44	2.5×10^{9}	2.1×10^{8}	1.9×10^{7}	2.5×10^{9}
L. reuteri 45	2.1×10^{9}	2.1×10^{8}	1.1×10^{7}	2.0×10^{9}
L. reuteri 12	2.6×10^{9}	2.1×10^{8}	1.6×10^{7}	2.6×10^{9}
L. reuteri 16	2.2×10^{8}	2.0×10^{7}	1.2×10^{7}	2.1×10^{8}

Pumpkin juice was supplemented with 1.5% NaCl

Table 3: Sensory evaluation (% of scale) of pumpkin juice fermented by different *L. reuteri* strains (°T 70 with 1.5% NaCl addition)

Strains	Taste	Aroma	Texture	Aftertaste	Ranking
L. reuteri 25	75	70	75	80	2
L. reuteri 42	65	60	70	85	4
L. reuteri 43	75	75	70	65	3
L. reuteri 44	55	65	80	80	4
L. reuteri 45	70	80	80	75	1
L. reuteri 12	45	55	65	50	6
L. reuteri 16	45	50	60	45	7
L. reuteri 19	50	60	70	55	5

Table 4: Influence of glucose, fructose, sucrose and their combinations on pumpkin juice fermentation (supplemented with 1.5% NaCl) with *L. reuteri* 42

	pH±SD (h)			OT±SD (h)		
L. reuteri 42	24	48	72	24	48	72
Control	4.23±0.02	4.01±0.02	4.01±0.02	69±1	91±1	96±2
1% glucose	4.76±0.02	4.35±0.02	4.16±0.02	40±1	68±1	83±2
2% glucose	4.75±0.02	4.27±0.02	4.20±0.02	35±1	64±1	74±2
4% glucose	4.75±0.02	4.20±0.02	4.20±0.02	32±1	62±1	74±2
1% fructose	4.84±0.02	4.36±0.02	4.27±0.02	42±1	62±1	77±2
2% fructose	4.82±0.02	4.36±0.02	4.23±0.02	40±1	50±1	69±2
4% fructose	4.76±0.02	4.36±0.02	4.21±0.02	39±1	49±1	66±2
1% sucrose	4.79±0.02	4.34±0.02	4.05±0.02	30±1	64±1	94±2
2% sucrose	4.78±0.02	4.36±0.02	4.12±0.02	32±1	64±1	78±2
4% sucrose	4.74±0.02	4.30±0.02	4.19±0.02	35±1	70±1	72±2
0.5% glucose+1% fructose	4.76±0.02	4.35±0.02	4.18±0.02	32±1	63±1	84±2
0.5% glucose+0,5% fructose	4.78±0.02	4.32±0.02	4.25±0.02	38±1	55±1	81±2
1% glucose+1% fructose	4.77±0.02	4.37±0.02	4.26±0.02	39±1	50±1	63±2
2% glucose+2% fructose	4.79±0.02	4.37±0.02	4.24±0.02	24±1	40±1	72±2

products, storage time and storage temperature (Shah, 2001), as well as affected by lactic acid produced during fermentation and storage stage and food matrix being fermented (De Vuyst, 2000).

It was observed that viability of different *L. reuteri* strains varied during storage of fermented pumpkin juice (Table 2). Thus, after 4 weeks of fermented juice storage *L. reuteri* 16 and 43 strains showed higher viability than other *L. reuteri* strains (Table 2).

Since organoleptic properties is the main characteristic of any food and beverage, pumpkin juice fermented with different *L. reuteri* strains was sensory evaluated to select the strains most suitable for the development of beverage.

Sensory evaluation of pumpkin juice fermented by different *L. reuteri* strains showed that the best beverage, taking into account all assessed parameters (taste, aroma, texture and aftertaste), was obtained by fermentation with strain *L. reuteri* 45 (Table 3).

Pumpkin juice was supplemented by additional carbon sources-glucose, fructose or sucrose in order to promote the growth of *L. reuteri* cultures. Surprisingly addition of glucose, fructose, sucrose and their combinations to the pumpkin juice rather inhibited the growth of *L. reuteri* 42 as acidification power during fermentation (24-72h) was lower as compared to control without sugars addition (Table 4). Concentration dependent effect of pumpkin juice supplementation with sugars was observed on titratable acidity. Therefore it could be concluded that pumpkin juice supplementation with sugars used does not promote fermentation process and is disadvantage.

In the study performed it was shown that application of certain *L. reuteri* strains for pumpkin juice fermentation is a promising way for development of novel non-dairy based probiotic fermented beverage.

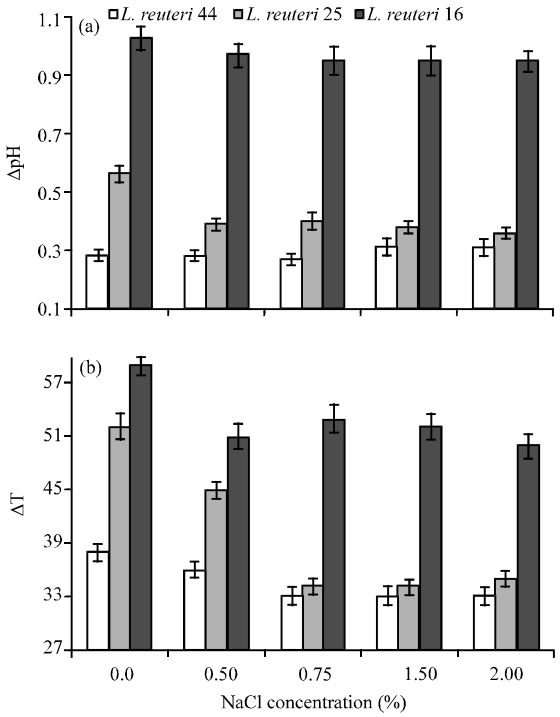

Fig. 2(a-b): Influence of different NaCl concentrations on, (a) pH and (b) Total acidity during the pumpkin juice fermentation (96 h) with *L. reuteri* 44

ACKNOWLEDGEMENT

The research was supported by ERDF Grant No. 2010/0322/2DP/2.1.1.1.0/10/APIA/VIAA/108.

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