ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Detection and survival of *Cronobacter sakazakii* and *Klebsiella pneumoniae* in naturally fermented black carrot beverage

Kajal*, Param Pal Sahota and Harminder Kaur Dhillon
Department of Microbiology Punjab Agricultural University, Ludhiana-141004, Punjab.

Manuscript Info

Manuscript History:

Received: 25 June 2014
Final Accepted: 26 July 2014
Published Online: August 2014

Key words:
Acidified vegetable products, black carrots, C. sakazakii, K. pneumoniae, traditional fermentation

Corresponding Author

Kajal

Abstract

The safety of traditionally acidified black carrot (*Daucus carota* L) vegetable products requires mandatory investigation of growth and survival potential of pathogenic bacteria in stored and fermenting black carrot beverage. The traditional beverage was found to be enriched in neutraceuticals with pH 3.1 , TSS(^{0}B) 3.4, titrable acidity(% lactic acid) 0.47%, brix: acid 8.51, total sugars 2.82%, reducing sugars 2.34%, ascorbic acid 480 mg/100ml, total phenols 39.60 mg/100ml and β-carotene 82.96 µg/l . With no sensorial defects in fermented beverage the microbiological analysis revealed the presence of Gram-negative pathogens (*Cronobacter sakazakii* strain WJ2275, accession number KF551983 and *Klebsiella pneumonia* strain HM2, accession number KF551984) capable of persisting during the initial stages of fermentation though they were quickly inactivated when analysed during the later stages of fermentation. Pathogens growth and survival was retarded as the pH decreased from 7.2 to 3.1 due to intense metabolism of bacterial strains but toxins produced by them may remain in the beverage as such. This contamination may have arisen as a consequence of treating soil with sewage sludge, manure and irrigation water. These studies have contributed to a better understanding of the behavior of emerging pathogens in acidified food products, thereby enabling the development of more effective strategies and interventions for its control like pre- pasteurization or development of consortia.

Introduction

Fresh vegetables like lettuce, parsley, cucumber, tomato and carrot are rich in vitamins, minerals and dietary fibers. Among all Black carrots (*Daucus carota* L.) are known for its high antioxidant property, due to its high vitamin C, anthocyanin and β-carotene content, nutraceuticals and biologically active compounds such as vitamins (A, D, K, Thiamine, Riboflavin, Niacine, Folic acid), dietary fibers and minerals (calcium, sodium, potassium, phosphorous and iron) (Alasalvar et al., 2001). Regular consumption of vegetables and its minimally processed products can reduce the risk of cancers, stroke, intestinal disorders and cardiovascular diseases.

Fermented foods are minimally processed products containing amylases, proteases and lipases that hydrolyse polysaccharides, proteins and lipids to non-toxic products with flavors, aromas and textures pleasant and attractive to the human consumer (Steinkraus, 1997). In India natural and traditional fermentation of carrots for beverage production has been followed simultaneously as natural lactic acid fermentation imparts probiotic properties to the minimally processed carrot beverage in which nutritional components of carrot are retained during storage. Lactic acid and acetic acid produced by lactic acid bacteria (LAB) during fermentation, acts as bio-preservative agents by disabling energy yielding and transport process of bacterial plasma membrane (Savard et al., 2002). LAB improves the bioavailability of minerals and trace elements by reducing the content of non-digestible material like cellulose, hemicellulose and polygalacturonic acid and reduces both the mutagenecity of intestinal

contents, serum cholesterol concentration that promote the activation of procarcinogenic compounds and enhances protein solubility and availability of limiting amino acids upto 50% (Nout and Ngoddy, 1997).

Though natural and traditional fermentation is a health boon but ubiquitous pathogenic bacteria are the main hindrance acting as source of contamination even in sterile conditions. Contamination arises as a consequence of treating soil with sewage sludge, manure and irrigation water (Hamilton et al., 2006; Heaton and Jones, 2008). The pathogens have ability to persist and proliferate in vegetables resulting in gastrointestinal outbreaks. Fresh vegetables contaminated with pathogens of Enterobacteriaceae family like *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Cronobacter sakazakii*.

Cronobacter sakazakii has been isolated from a wide range of foods including ultra high-temperature treated milk (UHT milk), cheese, meat, vegetables, grains, sorghum seeds, rice seeds, herbs, spices, fermented bread, fermented beverage, tofu and sour tea (Leclercq et al., 2002; Iversen and Forsythe, 2003). It is an opportunistic human pathogen that has been implicated in severe forms of septicemia (Lai, 2001), necrotizing enterocolitis (Van Acker et al., 2001) and meningitis (Bar-Oz et al., 2001), especially in neonates with mortality rate varying from 40-80% (Muytjens et al., 1988). The International Commission for Foods, due to the seriousness of pathologies with C. sakazakii has ranked the organism as "severe hazard for restricted populations, life threatening or substantial chronic sequelae or long lasting" (ICMSF, 2002).

K. pneumoniae (an important member of *Klebsiella* genus in Enterobacteriacae family), a food-borne opportunistic pathogen in fresh vegetables is lactose fermenting facultative anaerobe found in the normal flora of skin, mouth, and intestines, responsible for pneumonia (the destructive lung inflammation disease), urinary and lower biliary tract infections (Lopes et al., 2005; Ryan, 2004).

There is a little concern about survival of pathogenic microorganisms in traditionally fermented vegetable products and to our knowledge; there have been no documented outbreaks of food pathogens in acidified vegetable products. So, the aim of this study was to ascertain the safety of natural lactic acid fermentation for the consumption of traditionally fermented natural beverages by human beings.

2. Materials and methods

2.1. Preparation of Naturally fermented black carrot beverage

Black carrots (*Daucus carota* L.), procured from Department of Vegetable Science, Punjab Agricultural University, Ludhiana, Punjab, India were washed (with 0.01% KMS solution) , peeled and shredded under aseptic conditions. Shredded pieces and sterilized water (3 liters, boiled and cooled) were put in batch scale glass digester and heat treated (at 70-80^{0}C for 15 minutes), common salt and rye @ 2.75% (w/v) were added uniformly throughout the carrot pieces (Fig. 1). The digester was cotton plugged and the brine was allowed to ferment at room temperature (20±5^{0}C) for 5 days. During fermentation microbiological and physico-chemical parameters were analyzed at regular intervals.

2.2 Microbiological analysis

Beverage Sample for Microbiological analysis was serially diluted prior to enumeration on solid media. Bacteria were quantified after 24-48 h at 37^{0}C on tomato juice agar (TJA, pH 6.1±0.2, Himedia Laboratories Pvt. Limited, Mumbai, India) for lactic acid bacterial count, nutrient agar (NA, pH 7.0±0.2) for total bacterial count including contaminants.

2.3 Physicochemical analysis

The naturally fermented beverage was analyzed for TSS ($^{\circ}$B), pH, TDS, titrable acidity, total sugars, reducing sugars, total phenols, β-carotene and ascorbic acid content according to the standard methods. TSS of beverage was determined by using Erma hand refractometer of 0-32ºB (UNICO make). The pH value of beverage was determined using a digital pH meter (Electronic Corporarion of India Ltd., Hyderabad, type 101). TDS was determined using TDS meter (HiMedia's TDS-3). Titrable acidity on the basis of lactic acid was determined using the procedure of AOAC (1999). Total sugars of beverage samples was determined by phenol-sulphuric acid method of Dubois et al. (1956) and reducing sugars of beverage samples was determined by using method of Miller (1959). The titrametric method using 2, 6-dichlorophenol indophenol dye was used to estimate ascorbic acid (AOVC, 1996). Total phenols were estimated by method of Malik and Singh (1971). β- carotene was estimated according to the method of the Association of Official Analytical Chemists (AOAC, 1980).

2.4 Detection of pathogens in naturally fermented carrot beverage:

2.4.1 Phenotypic Identification of pathogens:

Naturally fermented carrot beverage was streaked on nutrient agar and tomato juice agar and incubated at 37^{0}C for 24 hours. Apart from probiotic strains, two pathogens *Cronobacter sakazakii* and *Klebsiella pneumonia* were presumptively detected and further identified morphologically (Gram staining, colony morphology, motility)

and biochemically (Catalase, oxidase, motility, methyl red (MR), Voges Proskauer (VP), citrate utilization , esculin hydrolysis and sugar fermentation)

2.4.2 Molecular identification of pathogens

2.4.2.1 Genomic DNA extraction and 16S rDNA gene amplification:

DNA was isolated using the method described by Schubert et al., 2008. The integrity and concentration of purified DNA was determined by agarose gel electrophoresis. The total G-DNA extracted was dissolved in sterile water (HPLC grade) and stored at 4 °C. DNA amplification was done by using 16S universal primer. The PCR (Peltier Thermal Cycler, BIO-RAD) reaction was performed in 100 µl volume (10 µl of 10x PCR buffer, 2.5 µl of dNTPs mix, 2.5 µl of each primer, 1 µl of Taq DNA polymerase) with 3 µl of DNA template. The amplification was performed with following programme: 5 minutes initial denaturation at 95°C, followed by 30 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 55°C, 1 minute extension at 72°C, and a final extension step of 5 minutes at 72°C. The amplified product was resolved in 1.2% agrose gel and visualized on gel documentation system (BIO-RAD, USA).

2.4.2.2 Sequencing of 16S rRNA Gene and Phylogenetic Analysis:

The purified 16S rRNA gene was performed using as a template in cycle sequencing reaction with fluorescent dye-labeled terminators (Big Dye, Applied Biosystems) of representative isolates of each cluster with same primer and run in 3130 XL ABI prism automated DNA sequences. The sequences were compared with 16S rDNA gene sequence available in the NCBI GeneBank database using BLASTn program. Identification to the species level was determined as maximum homology (≥96%) to a prototype strain sequence in the GenBank and phylogenetic analysis. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated through bootstrap analysis by MEGA 4.0 package (Tamura et al., 2007). The 16S rRNA gene sequences have been submitted to NCBI GeneBank database.

2.4 Studies on Survival of Cronobacter sakazakii and Klebsiella pneumoniae in fermented carrot beverage

Fermented beverage samples with 3.2 pH, TSS 3.4, titrable acidity 0.47%, total sugars 5.42mg/100ml, reducing sugars 2.19 mg/100ml, ascorbic acid 480 mg/100ml, total phenols 28.2 and beta-carotenes 82.96 µg/l were autoclaved at 120°C for 10 mins at 15 psi to inactivate the naturally existing bacterial population. *Cronobacter sakazakii* strain WJ2275 and *Klebsiella pneumoniae* strain HM2 isolated from naturally fermented beverage were used as inoculants in beverage samples and incubated at 4°C and 37°C for 1 week to evaluate the safety issues of natural fermentation which has been practiced by millions in Asia. The further studies were conducted to analyze the presence and survival of pathogens without any visual and sensorial defects in fermented beverage. To study the survival pattern of the pathogenic isolates, samples were taken at intervals of 0 hr, 1st, 3rd and 7th day of incubation. Both non-selective (Nutrient Agar) and selective (HiCrome Enterobacter Sakazakii Agar, Modified, pH 7.0±0.2 for *Cronobacter sakazakii* and HiCrome urinary tract infection (UTI) Selective Agar, HiVeg^{TM}, pH 7.2±0.2 for *Klebsiella pneumoniae*) media were used to detect any adverse effect of low pH and low temperature on their survival.

2.5 Statistical analysis

Triplicate trials were done for each experiment. Data were subjected for one-way analysis of variance using CPCS 1 software to determine if significant differences (P=0.05) existed between mean values of data.

Results and Discussion

Microbiological analysis of naturally fermenting black carrot beverage:

Microscopic observation revealed that the microfloral communities in fermented beverage included both Gram-positive and Gram-negative bacteria. The consortium of microorganisms which was either present at the start of fermentation process or developed during fermentation was analyzed. After serial dilution of brine, the diluents were spread on tomato juice agar, nutrient agar, carrot juice agar. Nutrient agar was used to quantized total bacterial population including contaminants, tomato juice agar was used to quantize specifically lactic acid bacterial population and carrot juice agar formulated during course of research work was used to quantize natural microflora of black carrots. The number of colonies that appeared on different medium used after 48 hrs of incubation were counted from a plate containing 100-300 colonies and number of microorganisms per ml of beverage was calculated as log number of viable cells (Fig 2).

The total bacterial count varied with time during fermentation of carrots on TJA, NA and CJA as 6.51, 5.56 and 3.42 log-cfu/ml respectively at the start of the fermentation and then increased exponentially to 8.63, 7.32 and 5.38 log-cfu/ml respectively up to 3 days. This increase was observed due to the presence of fermentable sugars in brine which were oxidized to lactic acid. With the production of lactic acid the pH and nutrient content started to drop down in which the persistent microflora failed to survive and showed a dip in their growth rate, thereby

displayed decline phase after 4th day of fermentation. Similar pattern of growth was observed by Malik and Garg (2011) during study of microbial succession in naturally fermented sliced carrots.

Physicochemical analysis

High TSS and total sugar content made carrots suitable for natural fermentation. Raw carrot juice and fermented beverage had pH 6.3 and 3.7 , TSS (°B) 5.8 and 3.4, titrable acidity 0.27%& 0.47%, brix- acid ratio 21.48 & 7.23, total sugars 6.25 & 2.82%, reducing sugars 3.85 & 2.34%, ascorbic acid 840 & 480 mg/100ml, total phenols and β-carotene 160 & 82.96 µg/l respectively (Table 1). Joshi et al. (2011) reported titrable acidity between 1.12-1.72%, pH 2.89, total sugars 38.52%, brix- acid ratio 31.25 of carrot based fermented appetizer.

Study of Effect of Fermentation time on physicochemical characteristics of naturally fermented carrot beverage:

The results of naturally fermented carrot beverage (Fig 3) showed that TSS of brine was increased up to 10 days, decreased thereafter and tended to stabilize up to 15 days during fermentation. The initial increase in TSS of brine attributed to the process of leaching in which the total solids including sugars eluted into the brine. The addition of salt to the carrot shreds during fermentation extracts out water and sugars besides other nutrients from the carrot shreds into the brine that might have provided a favorable growth medium for bacteria as reported earlier (Pederson and Albury, 1969). Thus, the extraction of sugars and its conversion into lactic acid is apparently responsible for the initial increase and subsequent decrease of TSS. The titrable acidity increased with the advancement of fermentation period up to 9 days of fermentation and decreased slightly thereafter. Similarly pH of beverage recorded a continuous decrease up to 10 days during fermentation and stabilized thereafter towards the end of fermentation. Apparently, the sugar extracted from carrot has been utilized by bacteria to produce lactic acid during fermentation which in turn increased the acidity and decreased the pH of beverage. After the complete utilization of sugars, constant acidity and pH were attained and the fermentation was considered complete. Similar trends were reported earlier in lactic acid fermentation of radish by Joshi and Sharma (2009).

Detection and identification of pathogens in naturally fermented carrot beverage:

The different phenotypic characteristics such as colony morphology (Fig 4), Gram reaction, Catalase, Oxidase, Citrate utilization, motility and sugar fermentation of isolated bacterial strains were studied. Both strains were Gram negative, Catalase negative, Oxidase negative, Esculin positive and had different ability to ferment sugar (Table 2).

rDNA gene sequence analysis:

The 16S rDNA gene sequences of two isolates have been submitted to NCBI Gene Bank database and the accession numbers assigned were KF551983 and KF551984. Comparison of sequences with those of the Gene Bank sequence database (www.ncbi.nih.gov) demonstrated that our sequences were collected from members of the genus *Cronobacter* and *Klebsiella*. Phylogenetic allocations were presented in Fig 5 (a) and (b). The 16S rDNA gel image was shown in fig 6.

Survival studies of *C. sakazakii* and *K. pneumoniae* in fermented beverage:

C. sakazakii and *K. pneumoniae* inoculated in fermented black carrot beverage after inactivating its naturally existing microflora showed initial bacterial count (0h) 5.32, 6.18 and 5.62 log-cfu/ml on nutrient agar (non-selective), Urinary tract infection (UTI) selective agar and *Enterobacter Sakazakii* Agar (selective) media respectively. Changes in bacterial population at 37 and 4^0C on 1st, 3rd and 7th day of incubation on NA, UTI and *Ent. Sakazakii* Agar was shown in Table 3, 4 and 5 respectively. This study revealed that log number of cells increased on 1st day and then decreased afterwards. Incubation at lower temperature i.e. 4^0C showed lesser growth as compared to higher temperature i.e. 37^0C. The reason for this difference in growth is the optimum growth temperature which is 37^0C. In strawberry, cabbage and carrot, *C. sakazakii* either did not change or gradually decreased at 4^0C. All fruit and vegetable juices except apple, strawberry, and cabbage juice supported growth at 25 °C. The highest populations were detected in watermelon juice (8.1 log CFU/ml) and carrot juice (7.3 log CFU/ml) stored at 30 h (Beuchat et al., 2009).

Effect of pH on growth of test bacteria:

The pH is the major antimicrobial factor in the fermented beverage and its effect on growth of pathogens is shown as Fig. 7 (a), (b) and (c). The highest population of pathogens was observed at pH 7 and the log number of cells decreased with pH drop. No growth was observed at lowest pH of 3.0 which is the final pH of fermented beverage. Same pattern of growth was observed on both selective and non-selective medium expect for selective medium for *C. sakazakii* in which no growth was observed at pH 4. This might be due to the reason of acidification which does not support the growth of pathogenic bacteria. Acidification reduced the concentration of *E. sakazakii* in different types of infant formula and vegetable based food products (Joosten and Lardeau, 2004; Richards et al., 2005; Coulin et al., 2006). In juices of vegetables, the reduction of pH after 48 h was correlated with a reduction of

the numbers of *E. sakazakii*, but with increasing numbers of *E. sakazakii* in juices of different fruits (Kim and Beuchat, 2005).

Table 1: Physicochemical characteristics of raw carrot juice and naturally fermented beverage

Physico-chemical Parameters	Raw Juice	Naturally fermented Beverage
pH	6.3	3.7
TDS (ppm)	2590	6830
TSS (^{0}Brix)	5.8	3.4
Titrable acidity (%lactic acid)	0.27	0.47
Brix- acid ratio	21.48	7.23
Total Sugars (%)	6.25	2.82
Reducing Sugars (%)	3.85	2.34
Ascorbic acid (mg/100ml)	840	480
Total phenols (mg/100ml)	53.9	39.60
Beta-carotene (µg/l)	160	82.96

Table 2: Morphological and Biochemical characteristics of *C.sakazakii* and *K.pneumoniae*

Characteristics	*C.sakazkii*	*K.pneumoniae*
Gram reaction	Gram –ve, short rods	Gram –ve, short rods
Colony characteristics	Yellow pigmented, round, entire margins	White, raised, mucoid, smooth texture
Catalase	-	-
Oxidase	-	-
Motility	Motile	Non motile
VP	-	-
MR	+	-
Citrate Utilization	+	-
Esculin Hydrolysis	+	+
Lactose	-	+
Maltose	+	+
Galactose	+	+
Raffinose	+	+
Mannose	+	+
Trehalose	+	+
Cellibiose	+	+

Table 3: Log number of cells/ml on Non-selective (nutrient agar) medium

	Days					
	1st		3rd		7th	
pH	37^0C	4^0C	37^0C	4^0C	37^0C	4^0C
	Log Number of cells/ml					

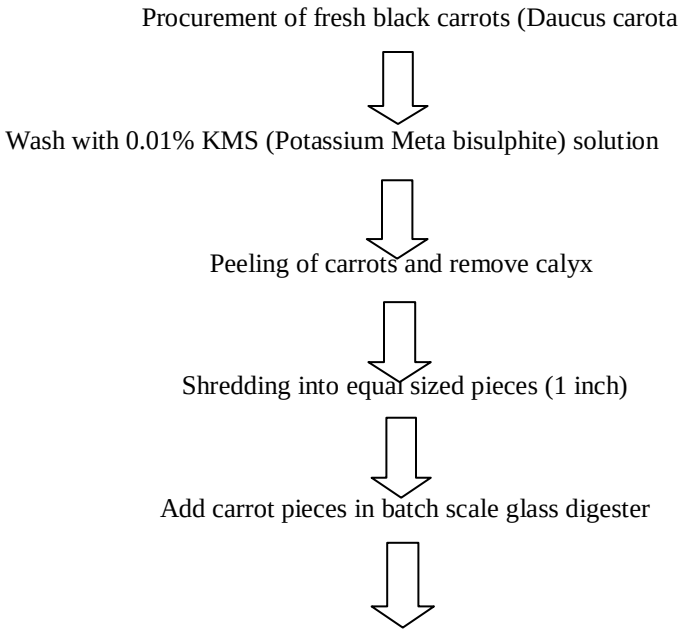
3	-	-	-	-	-	-
4	6.30	6.9	5.83	6.34	4.63	3.48
5	8.19	7.7	6.63	6.83	5.23	4.83
6	8.61	7.91	6.72	6.87	5.36	5.64
7	8.51	7.73	6.83	6.91	5.55	5.75
CD (5%)	**0.824**	**0.182**	**0.215**	**0.258**	**0.254**	**0.215**

Table 4: Log number of cells/ml on urinary tract infection (UTI) selective agar (selective medium for *K. pneumoniae*)

pH	Days					
	1st		3rd		7th	
	37^0C	4^0C	37^0C	4^0C	37^0C	4^0C
	Log Number of cells/ml					
3	-	-	-	-	-	-
4	6.48	7.28	5.58	5.08	4.56	3.30
5	7.96	7.71	6.0	6.67	5.21	4.60
6	8.12	7.40	6.44	6.41	5.58	5.08
7	8.23	7.48	6.72	6.62	5.69	5.52
CD (5%)	**0.258**	**0.163**	**0.838**	**0.165**	**0.215**	**0.183**

Table 5: Log number of cells/ml on *Enterobacter Sakazakii* Agar (selective medium for *C. sakazakii*)

pH	Days					
	1st		3rd		7th	
	37^0C	4^0C	37^0C	4^0C	37^0C	4^0C
	Log Number of cells/ml					
3	-	-	-	-	-	-
4	6.60	6.78	5.64	5.28	-	-
5	6.95	7.23	6.58	6.37	5.54	4.49
6	8.70	7.45	6.70	6.54	5.43	5.12
7	8.71	7.89	6.73	6.74	5.88	5.39
CD (5%)	**0.825**	**0.262**	**0.258**	**0.162**	**0.198**	**0.140**



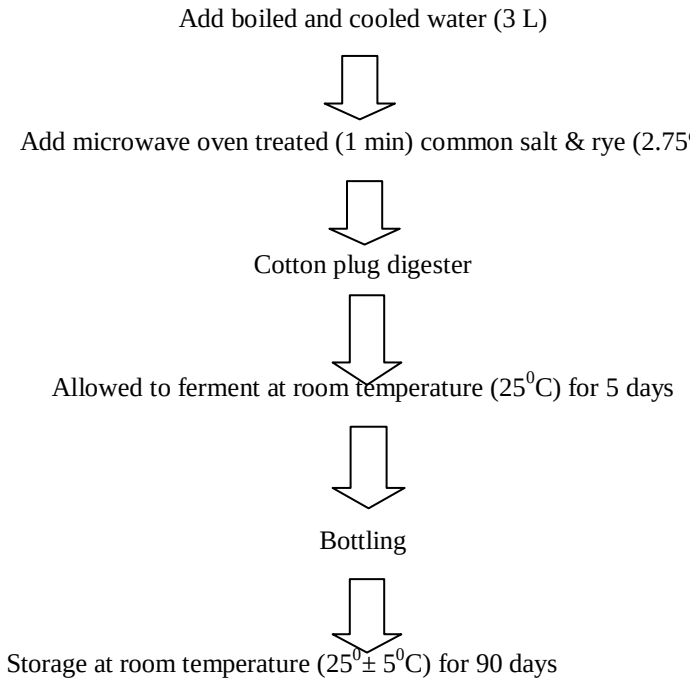


Fig 1: Flow chart showing preparation of naturally fermented black carrot beverage

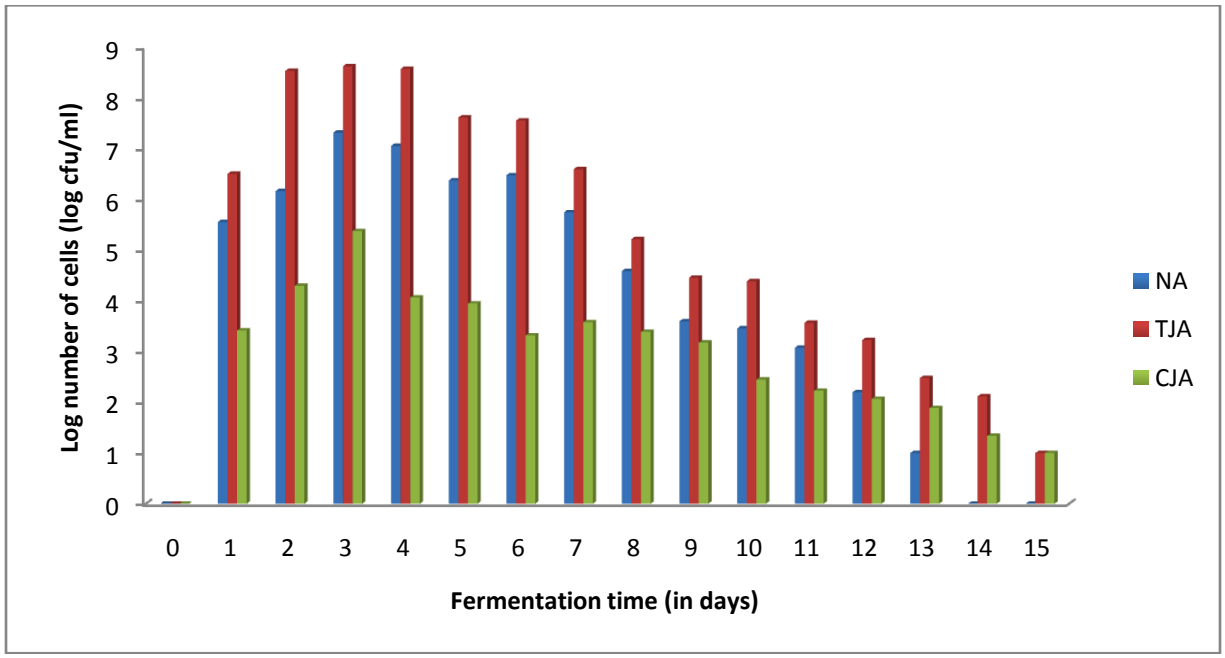


Fig 2: Changes in log number of viable cells during natural fermentation

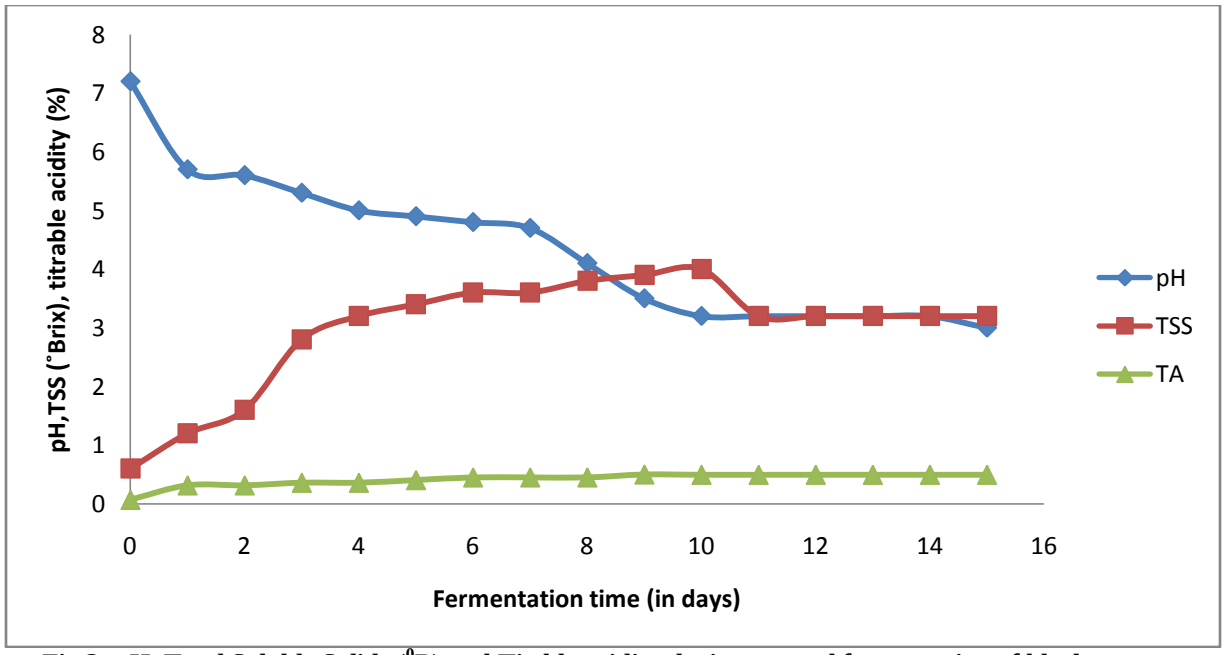
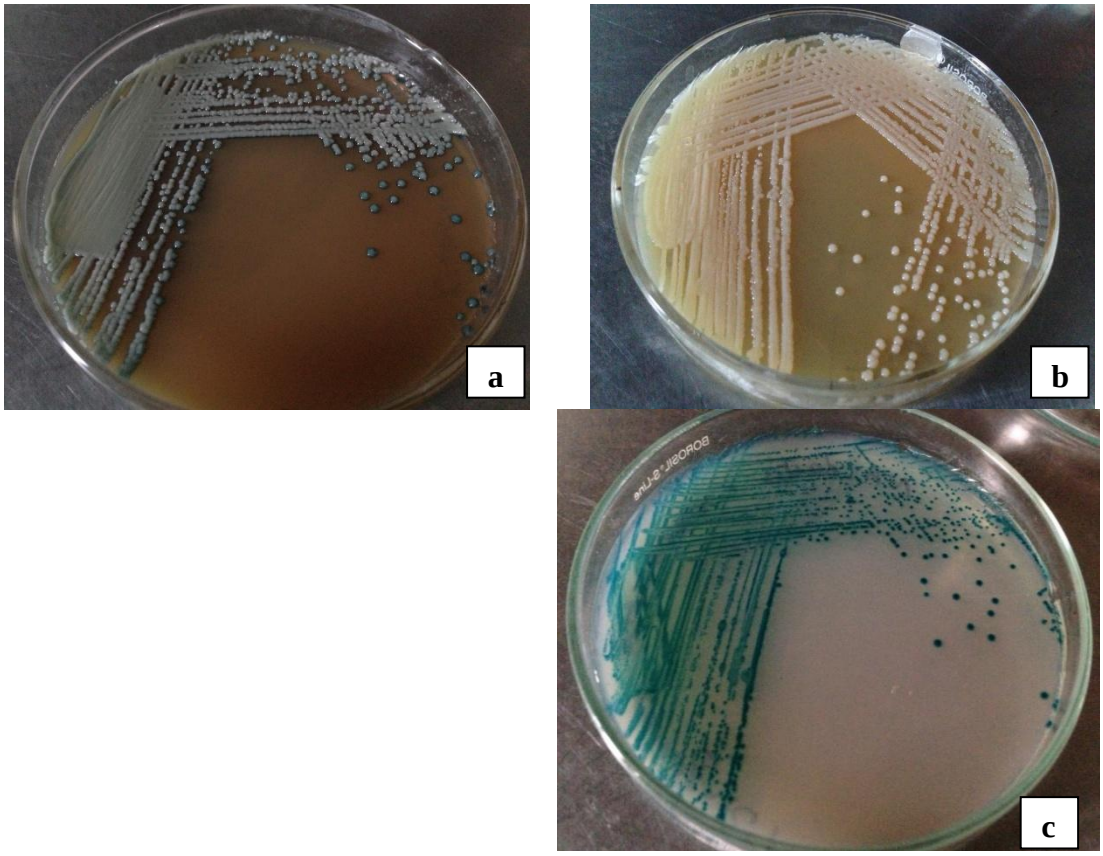


Fig 3: pH, Total Soluble Solids (^{0}B) and Titrble acidity during natural fermentation of black carrots



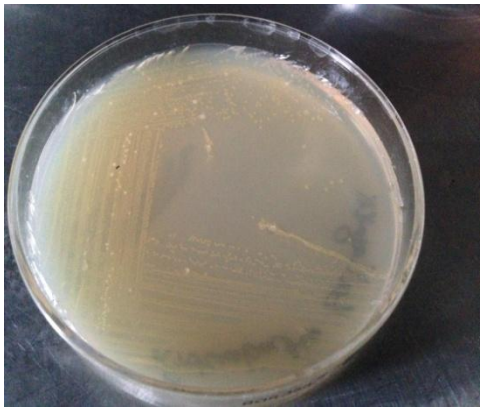


Fig 4: (a) & (b) shows growth of *K. pneumonia* on Selective (UTI agar) and Non-selective (Tomato juice agar) and (c) & (d) shows growth of *C. sakazakii* on Selective (*Enterobacter Sakazakii* agar) and Non-selective (NA) media

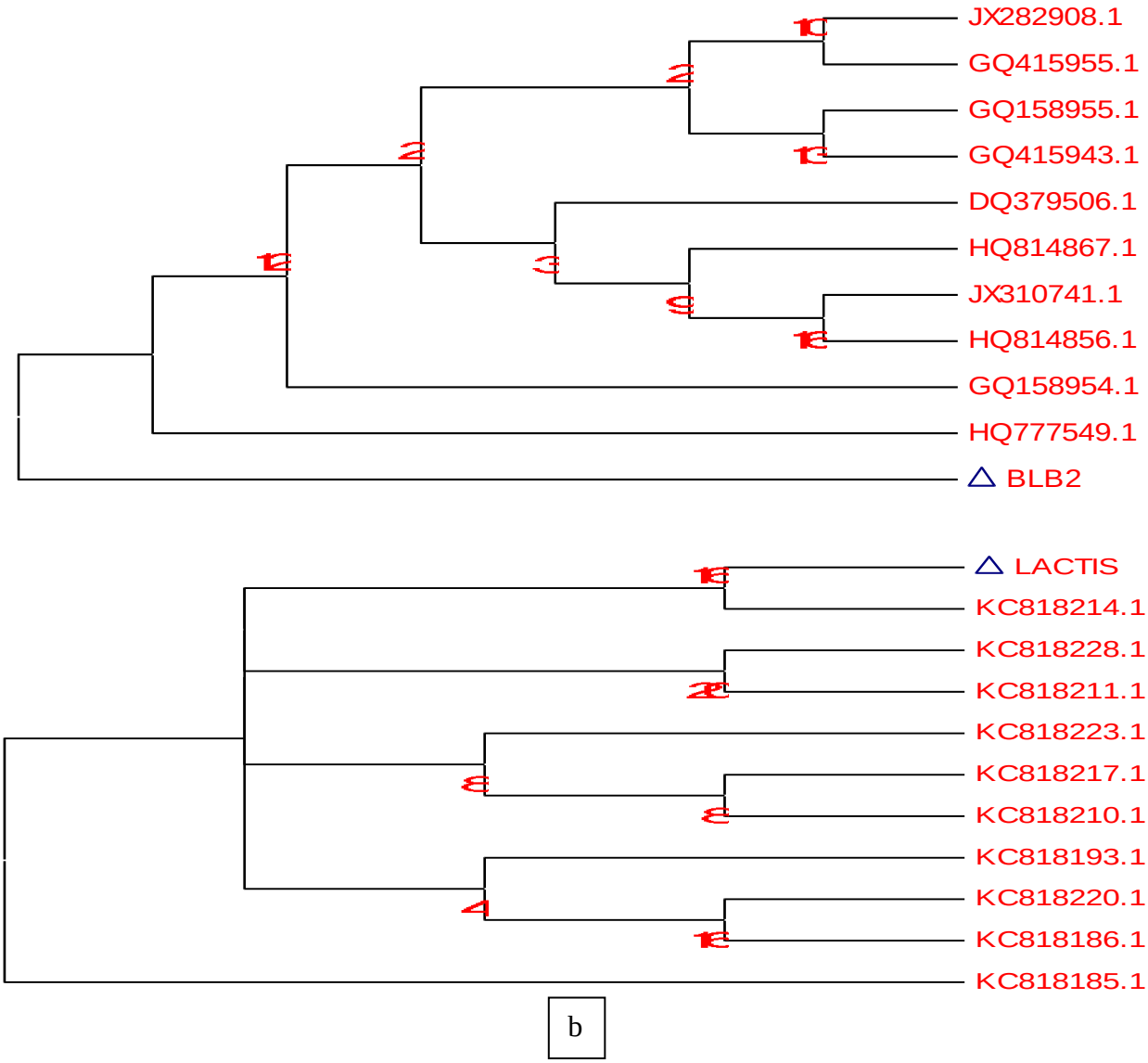
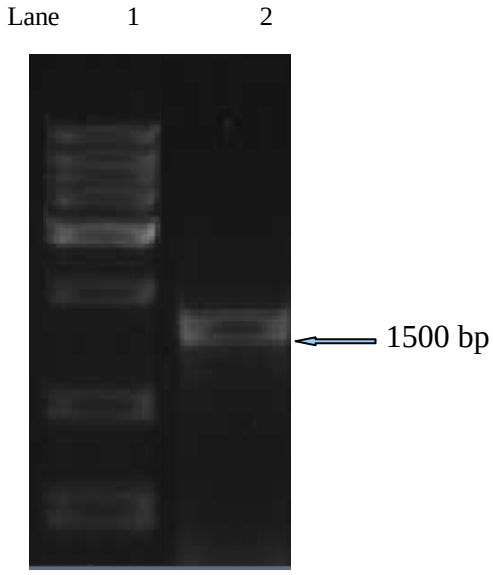
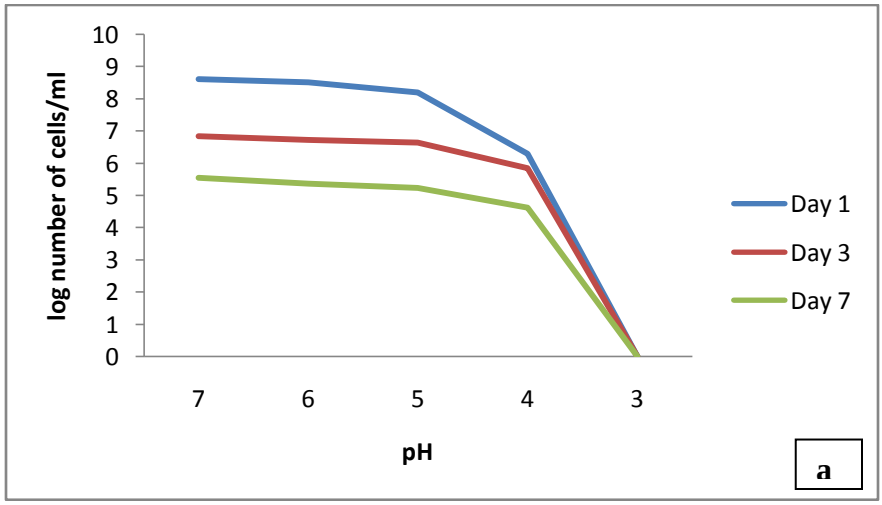


Fig 5 (a) and (b) : Phylogenetic tree showing evolutionary relationship between 11 Taxa of Genus *Cronobacter* and *Klebsiella* respectively



Lane 1: DNA marker
Lane 2: 16S rDNA amplicon band

Fig 6: Gel Image of 16SrDNA amplicon



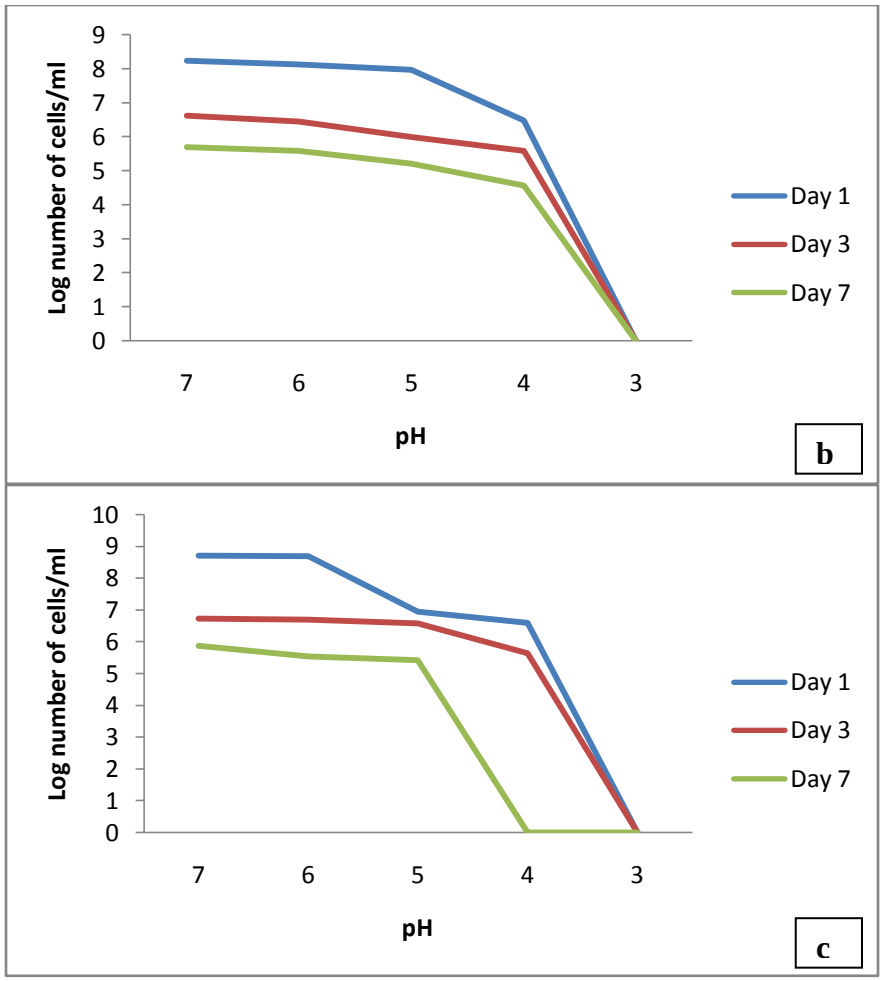


Fig 7 (a) (b) and (c) : Changes in log number of cells/ml with pH drop on NA, UTI and *Ent.Sakazakii* Agar respectively

Refrences:

AOAC (1980): Official methods of analysis. 13th ed. AOAC Washington DC: 504.

AOVC (1996): Methods of vitamin assay. AOVC Inc (Ed.) Interscience Publishers, pp. 306-12.

AOAC (1999): Official Methods of Analysis. 16th Edition,5thReversion,International, Gaithersburg, MD, method 942.15.

Alasalvar, C., Grigor, J.M., Zhang, D., Quantick, P.C. and Shahidi, F. (2001): Comparison of volatiles, phenolics, sugars, antioxidant vitamins and sensory quality of different colored carrot varieties. J. Agri. and Food Chem., 49:1410–1416.

Bar-Oz, B., Preminger, A., Peleg, O., Block, C. and Arad, I. (2001): Enterobacter sakazakii infection in the newborn. Acta Paedia., 90**:**356-358.

Coulin, P., Farah, Z., Assanvo, J., Spillmann, H. and Puhan, Z. (2006): Characterization of the microflora of attieke, a fermented cassava product, during traditional small-scale preparation. Int. J. Food Microbiol., 106:131–136.

Dubois, M., Gills, K.A., Hamilton, J.K., Roberts, P.A. and Smith, F. (1956): Colorimetric method for determination of sugars and related substances. Anal. Chem., 28**:**350-356.

Hamilton, A.J., Stagnitti, F., Premier, R., Boland, A.M. and Hale, G. (2006): Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water. Appl. Environ. Microbiol., 72:3284-3290.

Heaton, J.C. and Jones, K. (2008): Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. J. App. Microbiol., 104:613-626.

International Commission on Microbiological Specification for Foods (ICMSF) (2002): Microbiological Testing in Food Safety Management. Academic/Plenum Publisher 7, New York.

Iversen, C., Forsythe, S.J. (2003): Risk profile of Enterobacter sakazakii, an emergent pathogen associated with infant milk formula. Trends Food Sci. Technol., 14:443-454.
Joosten, H.M. and Lardeau, A. (2004): Enhanced microbiological safety of acidified infant formulas tested in vitro. S.A.J.C.N., 17: 87–92.
Joshi, V.K. and Sharma, S. (2009): Lactic acid fermentation of radish for shelf stability and pickling. Nat. Prod. Rad., 8: 19-24.
Joshi, V.K., Sharma, S. and Rana, N. (2011): Preparation and evaluation of appetizers from lactic acid fermented vegetables. J. Hill Agri., 2: 20-27.
Kim, H. and Beuchat, L.R. (2005): Survival and growth of Enterobacter sakazakii on fresh-cut fruits and vegetables and in unpasteurized juices as affected by storage temperature. J. Food Prot. 68: 2541–2552.
Lai, K.K. (2001): Enterobacter sakazakii infections among neonates, infants, children and adults. Med., 80: 113-122.
Leclercq, A., Wanequ, C. and Baylac, P. (2002): Comparison of fecal coliform agar and violet red bile lactose agar for fecal coliform enumeration in foods. App. Environ. Microbiol., 68: 1631-1638.
Lopes, A.C.D.S., Rodrigues, J.F. and Morais, M.A.D. (2005): Molecular typing of Klebsiella pneumoniae isolates from public hospitals in Recife, Brazil. Microbiol. Res., 160: 37-46.
Beuchat, L.R., Kim, H., Gurtler, J.B., Lin, L.C., Ryu, J.H. and Richards, G.M. (2009): *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. Int. J. Food Microbiol., 136: 204–213
Miller, G.L. (1959): Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.
Malik, C.P. and Singh, M.B. (1971): Extraction and estimation of total phenols, In: Plant enzymology and histoenzymology, Kalyani Publis., New Delhi, 286.
Muytjens, H.L., Willemse, H.R. and Jasper, G.H.J. (1988): Quality of powdered substitutes for breast milk with regard to members of the family Enterobacteriaceae. J. Clin. Microbiol., 26: 743-746.
Malik, K. and Garg, F.C. (2011): Microbial succession in naturally fermented sliced carrots. As. J. Food Ag.-Ind., 4: 359-364.
Nout, M.J.R. and Ngoddy, P.O. (1997): Technological aspects of preparing affordable fermented complementary foods. Food Con., 8: 279–287.
Netzel, M., Netzel, G., Kammerer, D.R., Schieber, A., Carle, R., Simons, L., Bitsch, I., Bitsch, R. and Konczak, I. (2007): Cancer cell antiproliferation activity and metabolism of black carrot anthocyanins Innovative. Food Sci. Emerging Technol., 8: 365–72.
Pederson, C.S. and Albury, M.N. (1969): Sauerkraut fermentation, Bulletein No. 824, NYSAES, Geneva, NY, 1-84.
Ryan, K.J. and Ray, C.G. (2004): Sherris Medical Microbiology (4th ed.). McGraw Hill. ISBN 0-8385-8529-9.
Richards, G.M., Gurtler, J.B., Beuchat, L.R. (2005): Survival and growth of Enterobacter sakazakii in infant rice cereal reconstituted with water, milk, liquid infant formula, or apple juice. J. Appl. Microbiol., 99: 844–850.
Saitou, N. and Nei, M. (1987): The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Bio. and Evo., 4:406-25.

Savard, T., Beaulieu, C., Gadren, N.J. and Champagne, C.P. (2002): Characterization of spoilage yeasts isolated from fermented vegetables and inhibition by lactic, acetic and propionic acids. Food Microbiol., 19: 363–73.
Schubert, M., Fink, S. and Schwarze, F.W.M.R. (2008): Field experiments to evaluate the application of Trichoderma strain (t-15603.1) for biological control of wood decay fungi in trees. Arboricul. J., 31**:** 249–268.

Steinkraus, K.H. (1997): Classification of fermented foods: worldwide review of household fermentation techniques. Food Con., 8: 311–17.
Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Bio and Evo 24:1596-1599.

Van Acker, J., de Smet, F., Muyldermans, G., Naessens, A.A. and Lauwers, S. (2001): Outbreak of necrotizing enterocolitis associated with Enterobacter sakazakii in powdered milk formula. J. Clin. Microbiol., 39: 293-297.