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Analysis of Volatile Compounds, Amino Acid Catabolism and Some Technological Properties of *Enterococcus faecalis* Strain SLT13 Isolated from Artisanal Tunisian Fermented Milk

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Authors' contributions

This work was carried out in collaboration between all authors. Author MZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Authors SM, RDD, EC and MY performed the analyses of the study and managed the literature searches. Authors PT and MH supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Enterococcus faecalis* strain SLT13, isolated from the Leben microbiota was investigated for their technological properties.
Place and Duration of Study: The study was undertaken in the LETMi laboratory, Tunis, Tunisia

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with collaboration with CWBI, Gembloux, Belgium, and at INRA (Unité de Biochimie Bactérienne), Jouy-en-Josas, France. The duration of the study was during the period of January 2012 to February 2013.

Methodology: *Enterococcus faecalis* SLT13 was identified biochemically and by ITS rDNA gene sequencing and then characterized for acidifying activity after growth in milk. Solid Phase Microextraction (SPME) and Gas Chromatography (GC) were used for analyzing the volatile profile of fermented milk obtained with SLT13. Catabolism of pheneylalanine and Leucine were studied through using radiolabelled amino acids as tracers. The rheological behavior of the fermented milk was determined using a viscosimeter.

Results: The final pH and acidity after growth of the strain SLT13 on milk were 4.29±0.1 and 75°D±2 respectively. The volatiles analyzed by SPME-GC-M S, included a wide range of volatile compounds: ketones, acids, alcohols, aldehydes, sulphur compounds and hydrocarbons.

The aminotransferase activity towards Leucine (290±95 nmol min^{-1} mg^{-1}) was higher than those towards Phenylalanine and Aspartic acid (63±29 and 37±13 nmol min^{-1} mg^{-1} respectively). In fermented milk containing radiolabelled Leucine, hydroxyisocaproate was the only metabolite produced by SLT13. The main Phenylalanine degradation product was phenyllactate and small amounts of phenylethanol and phenylacetate.

The milk fermented by *Enterococcus faecalis* SLT13 presented a consistency coefficient K= 0.156 and the flow behaviour index n=0.09.

This strain was suited for pilot-scale production and downstream processing with an important growth rate μ_{max}=0.9 h^{-1}, high viable count (1.19 10^{10} CFU ml^{-1}) and an important survival rate after freeze-drying (82%).

Conclusion: *Enterococcus faecalis* SLT13 exhibited remarkable technological properties and seems also suitable for pilot-scale production and downstream processing.

Keywords: Enterococcus; acidification; volatiles; aminotransferase activities; rheology; production.

1. INTRODUCTION

In recent years, increased attention has been paid to traditional dairy products because of their unique sensory characteristics due to the activities of autochthonous microbiota, derived from milk and the environment. Enterococci have been frequently found in traditional dairy products and reach high levels [1]. *Enterococcus* strains were isolated from fermented milk like Zabady, Laban and Rayeb [2]. The presence of *Enterococcus* species has been described in French cheeses derived from pasteurized and row milk, in Manchego chesse and Italian cheese, although *E. faecalis* and *E. faecium* are the most commonly isolated [3,4,5]. Generally, the presence of enterococci through cheese ripening affects positively taste, color and the sensory profile of the full-ripened cheeses [6]. The enterococci persistence or even their predominance in cheese might be attributed to their capability to grow over a wide range of temperatures, high tolerance to salt and acid pH and to produce proteolytic enzymes involved in casein degradation [1,7]. Several strains belonging to different *Enterococcus* species share interesting biotechnological traits by means of citrate metabolism, proteolytic and lipolytic activity [1,8,9].

Besides their positive traits, several risk factors are also associated with enterococci. Contrary to other lactic acid bacteria, enterococci are not considered as “Generally Recognized As safe” (GRAS). Some of the enterococcal strains are typical opportunistic pathogens that cause disease especially in the nosocomial setting, which may in part be linked to the presence of virulence determinants and antibiotic resistance [10].

Leben is a traditional fermented milk widely consumed in North Africa and in Middle Eastern countries [11]. The production process involves a spontaneous fermentation of cow milk at ambient temperature for up to 48 h. The fermentation is allowed to proceed until a firm coagulum is produced. The coagulum is then churned to separate Leben from butter. The product rich taste and consistency is similar to unsweetened commercial yoghurt or fresh cheese. The locally made naturally Leben is preferred by the local consumers to the fermented milk made using an imported starter culture. This difference is attributed to the presence of different microorganisms. The development of the typical flavour and texture was based on the autochthonous strains. Leben microbiota is

presented by lactococci as major group, but we can found lactobacilli and enterococci [12].

A detailed analysis of the technological properties of predominant bacteria of Leben, *Lactococcus,* has been performed [11,13,14]. However, no studies have been conducted to characterize the properties of enterococci isolated from Leben microbiota.

In this context, the purpose of this work is to study some technological aspects of *E. faecalis* strain (SLT13) isolated from artisanal Tunisian Leben: Acidification, volatile compounds production, amino acid catabolism, texturing potentialities, kinetical parameters, biomass production and survival rate after freeze drying.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Culture Conditions

The strain *Enterococcus faecalis* SLT13 used in this work was isolated from artisanal Leben. The strain was tested for Gram staining, catalase, mobility, oxydase, production of indole and H_2S. Sugar fermentation pattern of strain SLT13 was tested by the API 50 CHL system (BioMerieux, Marcy l'Etoile, France). DNA extraction and genotypic identification was determined on the basis of ITS-rDNA sequence according to Diop et al. in Centre Wallon de Biologie Industrielle (CWBI, Belgium) [15].

Enterococcus faecalis strain was cultivated either in M17 medium or in skimmed milk. Skimmed milk was reconstituted (RSM) with NILAC milk powder (NIZO, Ede, Netherlands) at 10% (wt/vol). The reconstituted milk was sterilized at 112℃ for 15 min and insoluble material was eliminated by centrifugation at 5,000 *g* for 30 min (SORVALL) before use.

The M17 medium was used for the bacterial growth in 20 l fermentor and for viable count (CFU ml^{-1}) (colony forming units). A colony from M17-agar was inoculated into a pre-culture medium in 250 ml Erlen Meyer flask filled to 80% by volume with M17 broth, where lactose has been substituted with 20 g l^{-1} glucose. This pre-culture (P1) was incubated at 30℃ for 16 h. The pre-culture P1 from the exponential growth phase was inoculated (200 ml) into 1 l Erlen Meyer flask filled to 80% with M17-glucose (pre-culture P2). Batch culture was performed in 20 l stirred fermenter (Biolafite - France) at 37℃. The pre-culture P2 (800 ml) in the exponential phase was used for the inoculation of the fermentor (16 l of culture medium). The temperature of the fermentor was maintained at 37℃ and the pH was controlled at 7±0.2 by the addition of NaOH (3N).

The *Enterococcus* cell concentration estimation was followed by using the turbidimetrical method (the optical density: OD) at 600 nm. Viable cells counting method was used to determine the number of cell. The specific growth rate μ_{max} was calculated from the slope of the regression equation of the linear portion of the curves relating log $OD_{600\ nm}$ to time, in hours. Glucose was analyzed by the Glucose Kit (Sigma, USA).

At the end of fermentation cells were harvested by centrifugation (Sorvall RC 3B, speed 500) at 7,200 *g* for 30 min and the cell biomass were supplemented with skimmed milk (6%) and glycerol (2%) as cryoprotectors than freeze-dried (Louw, model Epsilon 225D gefriertrocknungsanlagen GmbH, Germany). The survival rate after freeze-drying was calculated using the following equation:

*Survival rate (%) = (N/N') *100*

Where N is the cells number g^{-1} of dry matter after freeze-drying and N' is the cells number g^{-1} of dry matter before freeze-drying.

2.2 Acidification Ability

The acid production was tested by inoculating stationary culture of *E. faecalis* SLT13 (1% vol/vol) into reconstitute skim milk (RSM, 10% wt/vol). After incubation at 37℃ for 18 h, the titrable acidity was determined as degree Dornic (℗). 10 ml of culture were taken and titrated with sodium hydroxide solution (NaOH) N/9 in the presence of 1% (wt/vol) phenolphthalein indicator. The titrable acidity (℗) was calculated multiplying by ten the volume of NaOH used for titration. pH was recorded using a digital pH meter (H35010, HANNA Instruments, Ronchidivillafranca, Italy).

2.3 Rheological Measurements

The rheological properties of the fermented milk were measured according to Rao et al. [16] by using a viscosimeter (Rheomat RM180, Rheometric Scientific, Ville, Pays) in controlled rate mode. The sensor used was the coaxial cylinder system with a cylindrical rotor.

Fermented milk sample was gently stirred with a magnetic stirrer for 30 s at low speed to obtain homogeneous sample for rheological measurements. Samples of 65 ml were placed in the gap between the inner and outer cylinders. Samples were held in the sample cup until the temperature was stabilized before the measurements. A controlled ramped shear rate test was carried out to determine the rheological characteristics of the samples. The shear rate was increased linearly from 200 to 1200 s^{-1} (upward curve) and reduced back to 200 s^{-1} (downward curve). All viscosity values (η) and the resulting shear stress (ώ) were measured. Readings were expressed in Pa.s units.

The power law model was used to determine the consistency coefficient (K) and the flow behaviour index (n) of the samples using the shear stress data obtained from increasing shear rate measurements. Correlation coefficient for the model were higher than 0.98.

2.4 Citrate Utilization

The ability of strain to utilize citrate was evaluated by plating diluted culture on the Kempler and McKay medium (KMK). Plates were incubated at 30℃ for 48 h. Strains used citrate as carbon sources produced blue colonies while those that did not produced white colonies.

2.5 Analysis of Volatile Compounds by Solid-phase Micro Extraction (SPME) and Gas Chromatography-mass Spectrometry (GC-MS)

Analysis of volatile compounds was carried out according to Ziadi et al. [11]: Five millilitres of fermented milk by the strain *E. faecalis* SLT13 were added to a 20 ml glass sample vial (Perkin Elmer) and fitted with a self-sealing septum at its top, through which the solid-phase microextraction (SPME) syringe needle (bearing a fiber coated with 85 µm film thick carboxen-polydimethylsiloxane, Supelco, Bellefonte, USA) was introduced and maintain in the headspace at 60℃ for 30 min. The SPME fiber was conditioned according to the manufacturer's recommendations (280℃ for 30 min in the GC injector). The volatile analytes absorbed to the SPME fiber were analysed by GC-MS (Hewlett-Packard, 5890 SERIES II *plus*). The compounds were separated by a CPWAX-52CB fused silica column (50 m × 0.25 mm × 0.2 µm, Agilent, USA). The injection port was heated at 250℃, in splitless mode, and helium flow rate was maintained at 1 ml min^{-1}. The SPME fiber was maintained in the injection port for 10 min. The oven temperature program was held at 40℃ for 4 min, increased to 250℃ at a rate of 8℃ min^{-1} and held for 5 min. Detection was performed with the mass spectrometer operating in a scan mode (mass range from 35 to 400 m/z using a scan rate of 4.51 scans s^{-1}) and the ionization energy was set at 70 eV. The temperatures of the ion source and the quadrupole mass analyzer were held at 200℃ and 100℃, respectively. The eluted compounds were identified by their retention times and by comparison of their mass spectra with the Wiley Mass Spectral database (Wiley and Sons Inc., New York, USA).

2.6 Determination of Aminotransferase Activity

Cells grown in M17 to late log phase were harvested by centrifugation (10,000 *g* for 10 min at 4℃) and washed twice with 50 mmol sodium β-glycerophosphate buffer (pH 7.0). They were resuspended in 50 mmol potassium phosphate buffer (pH 7.5) containing 2 mmol 2-mercaptoethanol, 1 mmol EDTA, and 0.1 mmol pyridoxal 5'-phosphate (Sigma Chemicals, St. Louis, Mo.) and were disrupted with glass beads in a mini-beadbeater cell disrupter three times for 1 min each time, with 1 min of cooling on ice after each time. After centrifugation (14,000 *g* for 20 min at 4℃), the supernatants were filtered through 0.45 µm pore size filters (Millipore Co., Bedford, Mass.) and were considered cells extracts (CEs).

The protein concentrations of the CEs were determined by the Bradford method with the Coomassie protein assay reagent as specified by Pierce Chemical Company (Rockford, Ill.), with bovine serum albumin as the standard.

Aminotransferase activities towards Leucine, Phenylalanine and Aspartic acid in CEs were determined as previously described by Rijnen et al. [17]. Briefly, cell extract was incubated for 15 min at 37℃ in the reaction mixture containing 70 mmol Tris buffer (pH 8.0), 0.05 mmol pyridoxal phosphate, 10 mmol α-ketoglutaric acid (disodium salt, Sigma) and the amino acid substrate (3 mmol). Formation of L-Glutamate was measured by HPLC as described behind for free amino acid analysis.

2.7 Phenylalanine and Leucine Catabolism

Amino acid catabolism was studied during growth in milk according to Ziadi et al. [13]. Aliquots of 2.5 ml of skimmed milk containing 0.05 µmol of L-[2,3,4,5,6-^{3}H] Phenylalanine (100 to 140 Ci mmol^{-1}) or L-[4,5-^{3}H] Leucine (120 to 190 Ci mmol^{-1}) (Isotopchim, Peyruis, France) as tracer, were inoculated at 2% with M17 pre-culture of SLT13. The pre-culture was centrifuged and washed in sterile water before skimmed milk inoculation. The culture was incubated at 30℃ for 18h. Samples (200 µl) were taken before and after fermentation and 5µl of pure trifluoroacetic acid (TFA) were added. After keeping on ice for 5min, the samples were centrifuged 12,000 *g* for 5 min at 4℃. The soluble metabolites were then analyzed by reverse-phase HPLC (for Phe metabolites) or ion exclusion HPLC (for Leu metabolites) with both UV and radioactivity detection. Identification was made by comparison of retention times with those of appropriate standard compounds obtained from Sigma Chemical Co.

2.8 Statistical Analysis

All experiments were performed in triplicate and are reported as means ± standard deviation. The aminotransferases activities and the quantities of free amino acids degraded were subjected to a one-way analysis of variance using the general linear model procedure. The data obtained were compared using a two-sample comparison analysis and a t-test.

3. RESULTS

3.1 Strain Characterisation

In this work *E. faecalis* SLT13 isolated from artisanal Tunisian Leben made from cow milk was phenotypically characterized and investigated for different technological potentialities.

The phenotypic characterization of strain was resumed in Table 1. SLT13 was Gram+, catalase, do not produce gas from glucose and hydrolyze esculin. The strain was also tested for the ability to ferment carbohydrates. The identification was based, firstly on the results of routine biochemical tests. However, because of the low percentage of similarity obtained by sugar fermentation that could be originated due to the inadequacy selection of API CH50L for well identification of *Enterococcus* strains as it was reported by Diop et al. [15], the SLT13 phylogenetic analysis was based on the Internal transcribed spacer (ITS) rDNA region sequencing. The phylogenetic analysis was carried out by alignment of sequences consensus of genes ITS rDNA collected in the GenBank database (http://rdp.cme.msu.edu). An algorithm making it possible to calculate the percentage of homology of sequence carried out this alignment per pairs of nucleotide. SLT13 was to be classified as *E. faecalis*. The ITS rDNA sequence of *E. faecalis* SLT13 was available in the GenBank database of NCBI (National Center for Biotechnology Information) under the accession numbers GU017482. *E. faecalis* SLT13 grew and acidified in milk. The pH is 4.29 (75Ɗ) after 18 h of growth.

3.2 Volatiles Production

Volatile compounds including ketones, acids, alcohols, aldehydes, sulphur compounds and hydrocarbons from fermented milk with strain SLT13 are presented in Table 2. Although the SLT13 strain was unable to utilize citrate on Kempler and McKay (KMK) medium (Table 1), diacetyl and acetoin were detected in fermented milk.

The three most abundant acids were acetic, hexanoic and butanoic which arise from the fermentation of lactose and lactic acid. Hexanoic acid can be also formed from the lipolysis of free fatty acids. Two sulphur compounds were identified: dimethyl sulfide and dimethyl disulfide (Table 2).

3.3 Aminotransferase Activity and Phenylalanine and Leucine Degradation

The volatile compound profile analysis of milk fermented by SLT13 show the presence of compounds derived from amino acid catabolism namely benzaldehyde, 3-methylbutanal, diacetyl and acetoin. The transamination reaction is an important step in amino acid catabolism, the level of aminotransferase (AT) activity towards Phenylalanine, Leucine and Aspartic acid was investigated.

The strain SLT13 exhibited the highest level of activity towards Leucine (290±95 nmol min^{-1} mg^{-1}), this being due to Branched chain AT (BcaT). Activities regarding Phenylalanine and Aspartic

acid, due to Aromatic AT (AraT) and Aspartic acid AT respectively, were much weaker than on Leucine, 63±29 nmol min^{-1} mg^{-1} toward Phenylalanine and 37±13 nmol min^{-1} mg^{-1} toward Aspartic acid. Activity towards Leucine (due to both AraT and BcaT) was about 5-fold higher than activity towards Phenylalanine.

Table 1. Phenotypic characteristics and carbohydrate fermentation profiles of *Enterococcus faecalis* strain (SLT13) isolated from Tunisian leben

Characteristics	SLT13		
Gram reaction	+		
Morphology	Ovoid cocci		
catalase	-		
Mobility	-		
Indole production	-		
H_2S production	-		
Oxidase	-		
Acids from:			
Glycerol	+	Esculin	+
Erytrol	-	Salicin	+
D-arabinose	-	Cellubiose	+
L-arabinose	-	Maltose	+
Ribose	+	Lactose	+
D-Xylose	-	Melibiose	-
L-Xylose	-	Sucrose	+
Adonitol	-	Trehalose	+
α-Methyl-D-Xyloside	-	Inulin	-
Galactose	+	Melizitose	+
D-Glucose	+	D-raffinose	-
D-Fructose	+	Starch	+
D-Mannose	+	Glycogen	-
L-Sorbose	-	Xylitol	-
Rhamnose	+	β-Gentiobiose	+
Dulcitol	-	D-Turanose	-
Inositol	+	D-Lyxose	-
Mannitol	+	D-Tagatose	+
Sorbitol	+	D-Fucose	-
α-Methyl-D-Mannitol	-	L-Fucose	-
α-Methyl-D-Glucoside	+	D-Arabitol	-
N-acetyl glucosamine	+	L-Arabitol	-
Amygdaline	+	Gluconate	+
Arbutin	+	2-Keto-Gluconate	-
		5-Keto-Gluconate	-
Growth in milk	+		
pH end of fermentation	4.29±0.1		
Final acidity of milk (°D)	75.00±2.0		
Citrate utilisation	-		

+ = Positive reaction; - = negative reaction

In order to further deepen our knowledge about amino acid catabolism of the strain SLT13, the degradation of Phenylalanine and Leucine was monitored by using each radiolabelled amino acid as a tracer. Results are shown in Fig. 1. Even after 18 h, no degradation occurred in control milk in which cells were not included. The percentage of Leucine and Phenylalanine degraded to metabolites after milk fermentation with SLT13 were 66.41±2.1 and 36.99±1.32, respectively.

Table 2. GC-MS analysis of volatile compounds produced in fermented milk with the strain SLT13 (30 min sampling with Carboxen-PDMS fiber at 60°C)

Chemical family	Compounds	RT (min)
Ketones	2 Propanone	2.73
	2 Butanone	3.36
	Diacetyl	4.46
	2 Heptanone	8.72
	Acetoin	10.98
	Cis-1-decalone	16.41
Aldehydes	Acetaldehyde	2.33
	3 Methyl Butanal	3.52
	Benzaldehyde	15.09
Alcohols	Ethanol	3.76
Acids	Acetic	13.73
	Butanoïc	16.62
	Hexanoïc	19.71
	Octanoïc	22.45
Sulphur compounds	Dimethylsulfide	2.45
	Dimethyldisulfide	6.23
Hydrocarbons	Trimethylhexane	5.54
	Tetramethylpentane	8.03

RT: retention time in min

In fermented milk containing radiolabelled Leucine, hydroxyisocaproate was the only metabolite produced by SLT13 (Fig. 1). In fermented milk containing radiolabelled Phenylalanine, the principal metabolite produced was phenyllactate (75.57% ±3.45). The other metabolites produced in small amounts were phenylacetate and phenylethanol (Fig. 1).

3.4 Rheological Behaviour

The variation results of apparent viscosity (ηapp) versus shear rate (γ) of fermented milk by SLT13 show that the viscosity decreases with increasing shear rate forming a hysteresis loop (Fig. 2). The fermented milk is therefore non-Newtonian fluid, shear thinning and thixotropic and this is a common behavior with others fermented milk like kefir, Ayran, etc. The apparent viscosity value at a shear rate of 343 s^{-1} of Leben prepared with SLT13 was 0.038 Pa^{-1}. This behavior is common in other types of fermented milks and it's due to

casein precipitation during acidification, also exopolysaccharides producing strains can contribute to the viscosity of these products but in our study no exploration on EPS production by SLT13 was done.

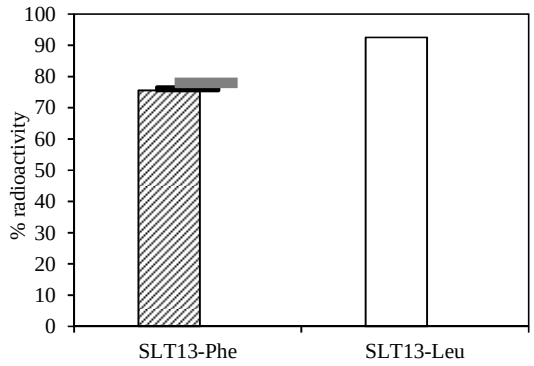

Fig. 1. Degradation metabolites of phenylalanine and Leucine by the strain SLT13 after growth in milk (▨ : phenyllactate, ■: phenylethanol, ■: phenylacetate and □: hydroxyisocaproate)

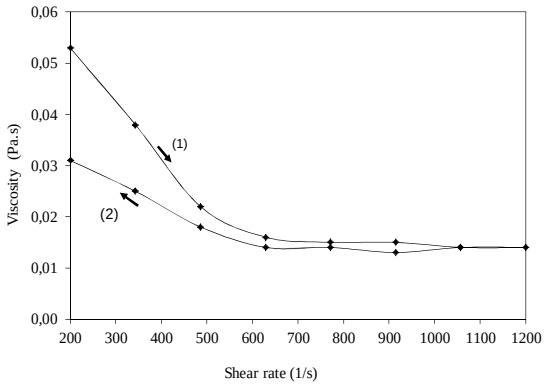

Fig. 2. Variation of apparent viscosity versus shear rate of fermenetd milk prepared with SLT13 as strarter culture: (1) charge, (2) discharge

3.5 Batch Culture and Survival Rate

The kinetics of growth of *E. faecalis* in M17 medium was analyzed (Fig. 3). Experiments in batch were conducted in a 20 l fully instrumented bioreactor. As shown in Fig. 3 there is a decrease of the glucose concentration to the profit of bacterial growth. At the end of batch culture (9 h) a complete degradation of glucose and a high viable cell counts (1.19 10^{10} CFU ml^{-1}) were noted. The decrease of OD_{600nm} at the end of fermentation can be due to autolysis and the inhibitory effect of the lactic acid and possibly of other metabolites. The survival rate after freeze-drying of the strain SLT13 was 82.00±5.00.

4. DISCUSSION

Several species of enterococci have been isolated and identified from cheese and fermented milk and many research works on their technological properties. The acidifying abilities of enterococci were, in general, low. Durlu-Ozkaya et al. [18] showed that enterococci, isolated from Beyaz cheese, lowered the pH of milk to < 5 after 24 h incubation and there is a tendency for the strains to become slow after 5 h. Similar results were obtained by Suzzi et al. [19] for *E. faecalis* strains isolated from artisanal Italian cheeses; pH lowering to about 4.5 after 24h fermentation of skim milk was obtained. Ayad et al. [2] has been showed that *Enterococcus* strains isolated from different Egyptian dairy products were poor acidifiers in milk. However, the poor acidifier strains can be used as adjunct cultures depending on their other important properties.

The proteolytic activity of dairy Lactic Acid Bacteria is essential for their growth in milk. Proteinase and peptidase activities in enterococci are generally low, with *E. faecalis* being the most actives species [6,20].

The volatiles profile of fermented milk by SLT13 shows that most of these compounds were found in other types of fermented milk: kefir [21], Lebanese Leben [22] and cheese [23]. The role of enterococci in flavour development is not clear. Some workers have reported that they cause deterioration in the flavour of cheese due to the high proteolytic activity that contributes to the bitter taste in cheese. Others authors believes that they play a major role in improving flavour development and cheese quality by the production of high amounts of acetate, acetone, acetaldehyde, acetoin and diacetyl [24]. Rea et al. showed no effect of enterococci on cheddar cheese flavour [25].

The strain SLT13 produces ketones, acids, alcohols, aldehydes, sulphur compounds and hydrocarbons. Most of the ketones are produced by microbial oxidation of fatty acids or by decarboxylation pathways [26]. 2-heptanone is well-defined product of the β-oxidation of free fatty acids [27]. Hydrolysis of milk fat by enterococci was reported, with *E. faecalis* and to a lesser extent *E. faecium* and *E. durans* being the most lipolytic species [6]. The esterolytic system of enterococci is more efficient than their lipolytic system. Enterococci show higher activity

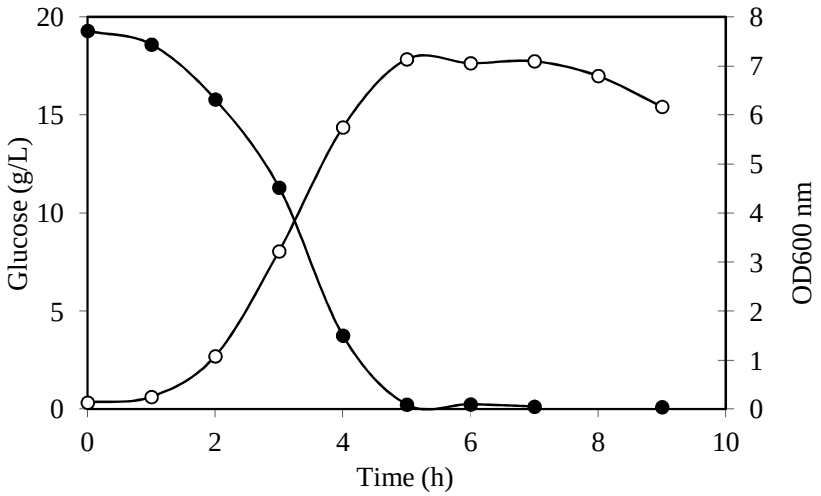

Fig. 3. Time course of OD_{600nm} and glucose concentration during batch culture of SLT13 strain on M17 in 20 l fermentor

than strains of most other genera of LAB with *E. faecium* being the most esterolytic species within enterococci [6].

Diacetyl and acetoin, result from glycolysis and citrate metabolism in *E. faecium, E. faecalis,* and *E. durans* [6,28,29]. Since that the strain SLT13 is enable to use citrate, these two compounds can also result from aspartic acid catabolism or from the active glycolysis witch takes place during the first day leads to pyruvate accumulation inside the bacteria cells; witch favours the production of acetolactate, the precursor molecule of acetoin and diacetyl [30]. These two compounds have aromatic properties distinct and significantly influence the fermented milk quality; in particular, diacetyl contributes to a buttery nut-like flavour of fermented dairy products.

Aldehydes generated were represented by acetaldehyde, benzaldehyde and 3-methylbutanal (Table 2). Acetaldehyde can be formed either by the metabolism of lactate or by the oxidation of ethanol [31]. Although many pathways led to acetaldehyde formation, Threonine deriving from protein degradation is considered the most important precursor of acetaldehyde. Benzaldehyde is generated from phenylanaine catabolism as demonstrated by the study of Tavaria et al. [32] on *Lactobacillus, Lactococcus, Leuconostoc,* and *Enterococcus* tested for their ability to catabolize free amino acids. Similar result was reported by Oumer et al. when *E. faecalis* was used as adjunct starter for Hispánico cheese [33].

The presence of 3-methylbutanal in the fermented milk made with SLT13 may be due to the amino-peptidolytic activity of enterococci. It can be derived from Leucine through Strecker degradation or via transamination [34]. Ethanol detected in fermented milk is a direct product of lactose fermentation (Table 2), but Tavaria et al. [32] showed that alanine can be a putative cursor for ethanol production.

Volatile sulphur compounds (Dimetheylsulfide and Dimetheyldisulfide), result mainly from methionine and cysteine catabolism. In lactic acid bacteria, methionine catabolism occurs mainly through a two step mechanism initiated by an aminotransferase that leads to the formation of 4-methylthio-2-oxobutyric acid which is subsequently converted to methanethiol which can be further converted to other sulphur compounds. Cysteine can be converted to methionine via a trans-sulphuration pathway [35].

Hydrocarbons may be produced during the autooxidation of milk fat. Flavor values for these compounds are influenced by many factors (number of carbon atoms, number and location of unsaturated double bonds, isomeric form and the nature of the food) but he overall flavor produced by autooxidation is the combination of many flavors imparted by individual volatile [36].

The level of aminotransferase (AT) activity towards Phenylalanine, Leucine and Aspartic acid was investigated. *E. faecalis* SLT13 shows higher aminotransferases activity. Similar results

were reported in our previous work: Two strains of *Lactococcus lactis* SLT6 and SLT10 isolated from Leben, exhibit higher aminotransferase activities toward Isoleucine and Leucine than Phenylalanine and Aspartic acid. The strain *E. faecalis* SLT13 degraded more Leucine than phenylalanine. This result can be explained by the higher level of aminotransferase activity towards Leucine. The only product of leucine degradation for the strain SLT13 was hydroxycaproate while SLT6 also produced 1–2% of isovalerate [13]. For phenylalanine, the compounds detected including phenyllactate, phenylethanol and phenylacetate are degradation products of phenylpyruvate which results from phenylalanine transamination [37]. Tavaria and Malcata [38] studied the AT activity towards Leucine, Valine, Methionine and Phenylalanine of *E. faecium* isolated from traditional Serra da Estrela cheese and showed that this strain is very active towards Leucine. In this work, the AT activity were determined in resting cells (0.0422 mmol.min^{-1} towards Leucine and 0.0086 mmol.min^{-1} towards Phenylalanine) but no AT activity was found in cells extracts.

Benzaldehyde detected in the volatile fraction of fermented milk was not detected in the HPLC analysis of fermented milk with radiolabelled Phenylalanine. This is may be due to the sensitivity of GC analysis.

The apparent viscosity value of milk fermented by SLT13 is higher than those reported by De Vuyst et al. [39] on fermented milk by strains of *Streptococcus thermophilus*. The value of consistency coefficient (K=0.156) and behavior index (n=0.09) are in the same range of attitude than those reported in the literature]. For example, Ayran, Turkish fermented milk, presented values of K varying between 0.125-0.009 Pa s^{n} and values of n between 0.940 and 0.583 [40,41]. Ao et al. [42] studied the fermentation characteristics of 53 strains lactic acid bacteria isolated from Xueo, Chinese traditional fermented yak milk and concluded that yogurt fermented with *E. durans* SCA2 acquired the highest viscosity and that fermented with *Lactobacillus paracasei* SCA33 showed the lowest viscosity. These authors reported higher viscosity values (>10^3 mPa.s). In deed, the viscosity of fermented milk is correlated to many factors: total solid content of the milk, interactions between bacteria and different proteins (spatial conformation, interactions, pH, and ionic strength), processing factors and exopolysaccharides producing strains [41,43].

The kinetic study of the strain *E. faecalis* SLT13 shows that the specific growth rate μ_{max} (0.90 h^{-1}) was in the same range of attitude of those reported in the literature. For example: *Lactococcus lactis* SLT6 (1.08 h^{-1}) isolated from Leben and cultivated in the same conditions, *Lactococcus lactis* NZ133 (1.10 h^{-1}) and 1.14 h^{-1} for *Lactobacillus bulgaricus* grown on complex media but higher than the 0.83 h^{-1} determined for *Lactobacillus delbrueckii* and 0.56 h^{-1} determined for *Lactococcus lactis* SLT10 grown also on complex media [14,44,45]. The total biomass yield based on lactose *Yx/s* (0.12 g g^{-1}) was in the same range that those reported in the literature. *Yx/s* range from 0.09 to 0.80 g g^{-1} for different species of LAB cultivated in similar media [46,47].

E. faecalis SLT13 presents a high survival rate. Kanmani et al. [48] studied the cell viability of *E. faecium* MC13 after freeze drying using different cryoprotectants and reported similar survival rates varying between 84.7±2.1% and 92.1±1.8%. This value is higher than those reported for the other LAB species varying between 20% for *Weisella paramesenteroides* and 51% for *Lactobacillus plantarum* [49]. Ayad et al. [2] have been demonstrated that enterococci isolated from Egyptian dairy products had a greatest ability to endure freeze-drying than the others LAB genera. The same authors have been showed that *E. faecium* was the most resistant strains. Carvalho et al. [50] studied the effects of three different growth media (MRS, M17 and Lee's) on survival during freeze-drying and subsequent storage of six strains of *E. faecalis* and two strains of *E. durans*. Their results showed that the majority of *E. faecalis* strains survived better after freeze drying when M17 or Lee's had been used as culture medium.

5. CONCLUSION

In conclusion, this study describes the technological potential of *E. faecalis* SLT13 isolated from traditional fermented milk Leben. Based on the overall evaluation of the results obtained, *E. faecalis* SLT13 has many important properties especially, flavour production, growth rate and fermentation yield. However, it must be emphasized that enterococci could be beneficial as adjuncts cultures for the improvement of organoleptic properties of dairy products but the emergence of enterococci resistant to glycopeptides and other antibiotics, the production of biogenic amines in some fermented foods, and the finding of a large variety of

virulence traits within both clinical and foodborne isolates raise questions about the safety of enterococci in foods. Thus, the screening of strains of enterococci with interesting technological properties for use in dairy industry could be based on the source of isolation, the absence of any possible virulence traits, and the lack of transferable antibiotic-resistance determinants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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