EMPLOYMENT OF FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY AS A METHOD FOR THE RAPID IDENTIFICATION OF LACTOBACILLI FROM SYRIAN WHITE CHEESE

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ABSTRACT

The identification of Lactobacilli has been mainly based on morphological, biochemical and other tests; however, this characterization is not reliable. In recent years, the use of infrared spectroscopy has been studied to differentiate bacteria, so this research aimed to identify the *Lactobacillus* bacterial strains using Fourier transform infrared (FT-IR) technique depending on the phenotype of viscous EPS after incubation strains at 30 °C or 45 °C for 3 days. The best discrimination between *Lactobacillus* was obtained with the following spectral regions: 1500-1200 cm^{-1} + 1200-900 cm^{-1} + 900-700 cm^{-1}, whereas the best discrimination between species was chosen as the following spectral regions: 2500-2000 cm^{-1} and 2500-3000 cm^{-1}. Our results indicated a good discrimination for the genus and species level, and sometime up to the subspecies level. The spectral database allowed us to identify new strains at the genus and species level for the collected samples. In conclusion FT-IR technique has afforded to identify strains within a period not exceeds 24 hours and can be used as a fast and reliable identification method for lactobacilli strains.

Key words: *Lactobacillus*, API System, lactic acid bacteria, starters, isolates, spectral region, fermentation.

INTRODUCTION

Syria has several types of cheeses that form an integral part of its native cuisine. There is an old folk saying that cheese was born from a serendipitous discovery by the Bedouins of the Arabian Peninsula. White cheese is the most popular cheese in Syria and makes up about 60% of the produced and consumed cheese in the country. It differs specially from other pickled cheese in that the milk is salted at the first step of its production.

Lactic acid bacteria "LAB" are extremely distributed in nature. The lactic acid fermentation, which these bacteria perform, has been known for a long time and applied by humans for making different foodstuff Barakat et al., (2011). The identification of Lactobacilli has been mainly based on morphology, gram staining and fermentation of carbohydrates methods, which are currently still being used. However, the characterization of some *Lactobacillus* species by biochemical tests alone is not reliable Alrubaye et al., (2018), because of the great variations in biochemical features between strains which are currently considered to the same species. In fact, some species are not easily distinguishable by phenotypic characteristics Coeuret et al., (2003). In recent years, the taxonomy has changed considerably with the increasing knowledge of phylogenetic relationships and the genomic structure between *Lactobacillus* spp. Zheng et al., (2017); Chung et al., (2018). This novel taxonomy based on DNA analysis offers a variety of advantages over other conventional typing procedures, such as the capacity to identify bacteria at the strain level, the stability of the genomic DNA analysis, and the ability to automation and statistical analysis Lee et al., (2016).

The use of infrared spectroscopy to differentiate bacteria has been studied since the 1950's Amiel et al., (2000). For about ten years, the development of modern interferometric infrared spectroscopy, Fourier transform techniques and efficient low-cost

computers have given a new impulse to this research field Faghihzadeh et al., (2016); Mura et al., (2012); Wenning et al., (2006). Bacteria spectra are usually recorded in the mid-infrared. They are specific to one bacterial strain and show the vibrational characteristics of all the cellular components: fatty acids, intracellular and membrane proteins, polysaccharides, nucleic acids. Statistical treatment of spectral data allows distinguishing between different genera, species and even strains. That is why more research teams are interested in FTIR characterization of bacteria Savic et al., (2008). The aim of this study was to characterize *Lactobacillus* isolated from Syrian white cheese using physiological, genotypic and phenotypic methods, and to describe the use of FTIR spectroscopy in discriminating some species of *Lactobacillus.*

Materials and Methods

White cheese samples

Fifty-six samples of white cheese have been collected from markets of different Syrian towns between October 2010 and October 2011. Samples were picked in sterilized bags, kept in ice boxes and transported to the laboratory for analysis.

Isolation of Lactobacilli

Ten grams of samples were homogenized with 90 ml of sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 100 ml distilled water, pH= 7.0). The homogenate was serially diluted and the appropriate dilutions were surface plated on MRS agar media (MERCK, Germany). The plates were then incubated at 30 °C or 45°C for 3 days.

Physiological and biochemical tests

Isolated bacterial samples were tested for gram reaction, catalase production, spore formation and cell morphology according to the methods described by Kebede et al., (2007). The growth in the presence of 4% and 6.5% NaCl was observed in MRS broth (MERCK, Germany) at 30°C for 2 days, and the production of acetone from glucose was

determined using Voges-Proskauer test Alrubaye et al., (2018). The production of CO_2 from glucose was noted in MRS broth containing inverted Durham tubes Marroki et al., (2011).

API assay

The API 50CHL (Biomerieux, Marcy l'Etoile France) was used to identify the enzymatic and carbohydrate fermentation patterns of *Lactobacillus* strains. The API of all isolates were overnight cultured in MRS broth then added individually to the substrate in wells of the API strips. The inoculated strips were incubated at 37°C and then monitored for changes in the color of medium after 24 h. Discrimination between isolates was based on the principle of a pattern matching manual as described by the manufacturer.

FTIR measurements

1- Preparation of the samples: Samples were performed according to the methods of Alvarez-Ordo´n˜ez et al., (2010), Burgula et al., (2006) and Carlos t et al., (2011). The 96 isolates stored at 80°C were streaked and subcultured tryptone soy agar plates (TSA containing 15 g tryptone, 5 g soya peptone, 5 g sodium chloride and 15 g agar per litre, Oxioid, Basingstoke, United Kingdom) for 24 h at 30°C. Colonies were transferred from the agar plate to an infrared-transparent ZnSe sample holder (25 mm in diameter) by replica stamping and were dried to a transparent film under mild vacuum (2.5–7.5 kPa), with a controlled atmosphere of 5–10% relative humidity for approximately 30 min. The spectra were inscribed and evaluated according to Davis and Mauer, 2011.

2- Phenotypic typing of FTIR spectra: Samples were run in replicate and analyzed by FT-IR spectroscopy using a TENSOR spectrometer (Bruker Optik, Karlsruhe, Germany) in transmittance mode. All the spectra were recorded in the region between 4000 and 500 cm^{-1} with an IFS 28/B Fourier transform infrared spectrometer (Bruker Optik, Karlsruhe, Germany). Nominal physical resolution was set to 6 cm^{-1}, a Blackman/Harris apodization was used for Fourier transformation and a zerofilling factor of 4 was applied to yield an encoding interval of approximately one data point per wave number. The quality of each spectrum was evaluated using a quality test in the OPUS 5.0 software.

Statistical analysis.

The data were analyzed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA), a chi-square test and Fisher's exact two-tailed test analysis were performed at the 5% level ($p < 0.05$).

Results

From the collected samples, a total of 96 isolates of *Lactobacilli* were classified as rods gram positive, catalase–negative, were found to be long bacilli with rounded ends. They are often found in pairs or chains of varying length or single cells and these could tentatively be identified as derivatives of this genus. (Table 1).

Results of API 50CHL

A total of 96 isolates were screened for their performance regarding growth characteristics on carbon sources supplied by the manufacturer. Based on phenotypic characteristics and interpretation of the API database, 35 isolates (36.45% from isolates) were identified as *Lb. casei*. 28 isolates (29.16%) were identified as *Lb. plantarum*, 21 isolates (21.87%) were identified as *Lb. lactis* and 12 isolates (12.5%) were identified as *Lb. bulgaricus*. Figure (1) and Table (2) revealed the distribution of *Lactobacillus* isolates from Syrian white cheese.

The statistical study indicated that no relationship was found between white cheese and *Lactobacillus* isolates among the sampling regions during the study period (unpublished results).

FT-IR results

Results obtained with the FTIR identification methods are shown in Figures (2-4). Out of 96 isolates purified on tryptone soy agar plates, were found identical to selected type strains by API system and *Lb. bulgarcius* (Hansan) strain which used as a positive reference.

Discussion

In the present study, the distribution of *Lactobacillus* was revealed by using classical and DNA techniques and the use of FTIR spectroscopy to differentiate some species of *Lactobacillus*. *Lactobacillus* is among the dominant members of the microflora of many types of cheeses. The milk can be curdled fresh (never after pasteurization) with the addition of two starters Barthelemy and Sperat-Czar, (2004). The cheese can be consumed either fresh or after conservation in salted water, or alternatively frozen at -18°C up to 6-8 months. The Syrian cheese comes in many different kinds; one of which is sort of like a braided mozzarella. The predominance of *Lactobacillus* is most likely due to the warm climatic condition of the production regions Cueto et al., (2007), and perhaps to high acidity. Similar results were obtained by Hamza et al., (2009) who found that the isolates that obtained from Sudanese sour milk (Rayeb) were identified as *Lactobacillus*, in general. The same results were found by Abd El Gawad et al., (2010) in traditional Rayeb Milk in Egypt. One sees that the majority of the isolates are *Lb. casei*. A relatively large amount of *Lb. plantarum* is found principally in milk and curd, presumably due to the survival rate of these strains at temperatures above 50° C which is lower than it for *Lb. casei* Weinrichter et al., (2001). In this study, we mentioned that the phenotypic identification of the isolates would not be positively identified solely by means of microscopic observations of cellular morphology, but must be associated with other methods like as API systems, in particular. However, phenotypic characterization based on sugar fermentation tests may not always provide sufficient basis for the reliable identification of LAB, as reported by other researchers Nigatu, (2000); De Angelis et al., (2001); Muyana et al., (2003). The inability to use PCR for all isolates is related to the absence of their genes sequencing.

Although the FTIR spectra of the four principal FHL species are very similar as shown in Figures 2-4, the small observed differences were sufficient to identify the majority of the strains employing technique used for the identification of chemicals.

The best discrimination between *Lactobacillus* was obtained with the following spectral regions: 1500-1200 cm^{-1} + 1200-900 cm^{-1} + 900-700 cm^{-1}. Based on the results of other teams Cur et al., (1994); Savic et al., (2018); Lefier et al., (2000), the observed variation in different regions of the FTIR spectra, and the rather significant region of 1600 – 1800 cm^{-1}; the following spectral regions were chosen in order to obtain the best discrimination between species in this study: 900-1800 cm^{-1} and 2800-3100 cm^{-1} Amiel et al., (2000).

Moreover, we can notice that the *Lactobacillus* species are well discriminated. The relevant spectra of these species show clear differences, particularly in two regions: the band at 1740 cm^{-1} characterizing CO stretching vibration of the ester functional groups of the phospholipids, and the region 1200-900 cm^{-1} characterizing oligo and polysaccharides COC and COP stretching vibration, known to be selective at the species and strain levels Mariey et al., (2001); Oust et al., (2004).

Ninety-six isolates of *Lactobacillus* were tested in FTIR libraries. FTIR identification of strains tallies with previous identification (biochemical methods) in 100% of the cases at the genus level and for species level. This study was to specify the ability of FTIR to discriminate and identify *Lactobacillus* involved in the white cheese industry. Our results indicate a good discrimination at the genus and species level, even at the subspecies level. The best spectral regions have been determined for each genus. The spectral database elaborated allows us to identify new strains, with a good percentage of correct results 100% at the genus and species level for collection strains.

In pastoral societies, milk is traditionally consumed predominantly in the form of white cheese. In many areas, cow or ewe plays a central role as milk suppliers where they are either home-consumed or sold De Angelis et al., (2001). To prepare fermented cow or ewe milk, containers of plastic, clay pots, plant fiber vessels or hollowed wood vessels are used. The daily residual fresh milk is poured into the milk container and the added starter is yogurt from ex-day. The milk is left in a quite warm place normally between 40 and 45°C, in a covered container sheltered from dust for usually 4 h until it becomes sour then it is stored in a refrigerator until used.

Due to the spontaneous nature of the fermentation, this traditional method results in a product with varying taste and flavor Brennan et al., (2002). To improve the spontaneous traditional fermentation, controlled fermentation using lactic acid bacteria starter culture is a very important strategy for milk processing. Since new lactic acid bacterial starter cultures with an industrially important functionality are being developed, it would be very essential to search novel strains among traditional food stuff which can contribute to the microbial safety and or offer one or more organoleptic, technological, nutritional, or health advantages Mufandaedza et al., (2006). Lactic acid bacteria that can produce sugar polymers, antimicrobial substances, aromatic compounds, sweeteners, vitamins, useful enzymes and have probiotic properties could constitute an important step which helps in the promotion of food industry Jose et al., (2007).

Conclusion

Lactic acid bacteria "LAB" are extremely distributed in nature. The identification of Lactobacilli mainly based on morphology, gram staining and biochemical tests which are not reliable, for this reason we used FTIR for characterization of LAB. Our results indicated a good discrimination for the genus and species level, and sometime up to the subspecies level. FT-IR technique has afforded to identify strains within a period not exceeds 24 hours and can be used as a fast and reliable identification method for lactobacilli strains.

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Table 1: Morphological, cultural and physiological characteristics of the isolate

Test		Isolates
No. of isolates		96
Cell Shape		Rod
Gram stain reaction		G+
Spores formation		-
Catalase activity		-
CO_2 from glucose		73
VP		56
Growth in a medium with NaCl (%)	4	96
	6.5	75

Table 2. Fermentation of some carbohydrates by *lactic acid* bacteria isolated from yogurt by using API system.

No. of isolates	Bacteria	API system (carbon sources)											
		ESC	GAL	SOR	LAC	GLYG	AMD	RAF	INU	TRE	RIB	VP	GLU
12	*Lb. bulgaricus*	-	+	-	+	-	-	-	-	-	+	+	+
21	*Lb. lactis*	-	±	+	-	-	+	+	-	-	-	-	-
35	*Lb. casei*	+	+	+	+	+	+	+	+	-	-	-	-
28	*Lb. plantarum*	+	+	-	+	-	-	-	-	+	+	+	+

+: positive fermentation; -: negative fermentation; ±: partial fermentation.

ESC: esculin; GAL: galatosidase; SOR: sorbitol; LAC: lactose; GLYG: glocogen; AMD: amidon; RAF: rafinose; INU: inulin; TRE: trehalose; RIB:ribose; VP: vosges-proskauer; GLU: glucose.

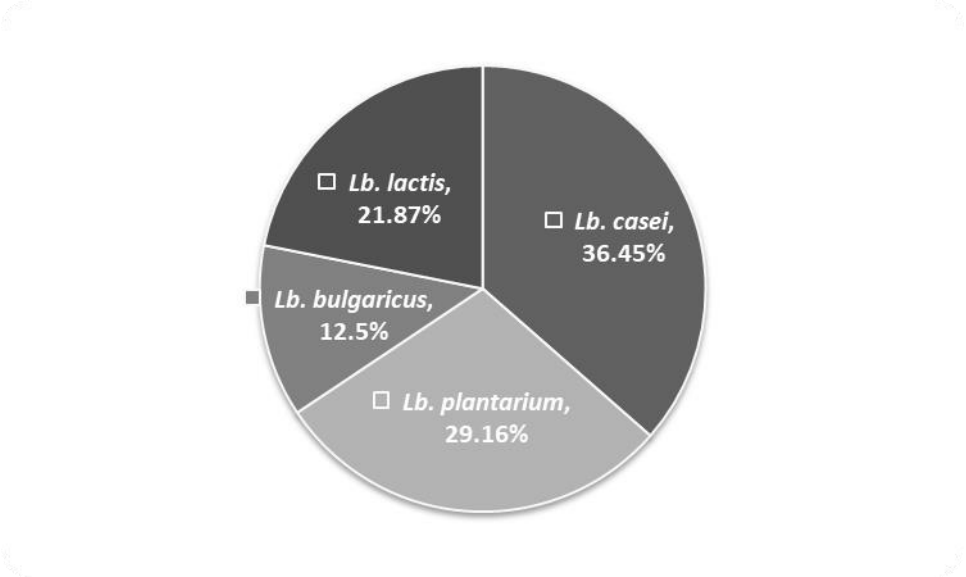

Figure. 1. Percentages of *Lactobacillus* isolated from white cheese

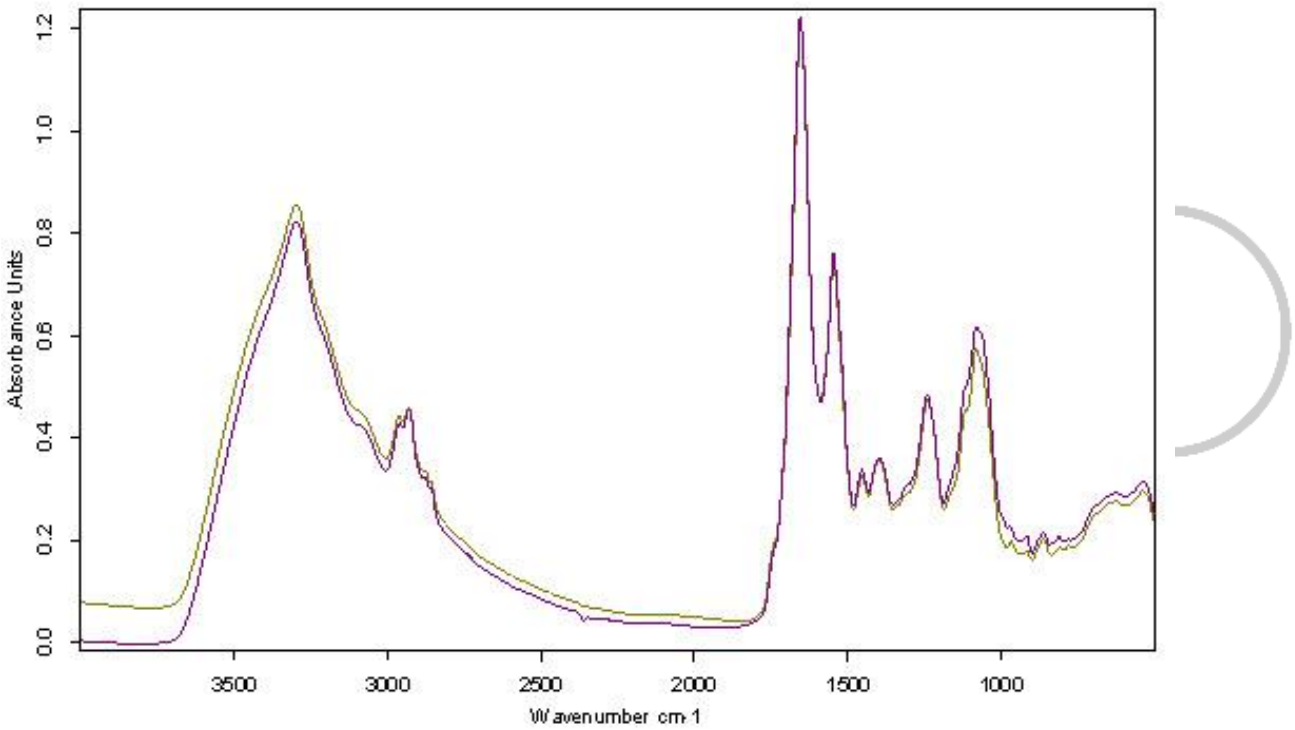

Figure. 2. Average FTIR spectra of *Lb. casei* and *Lb. bulgaricus*

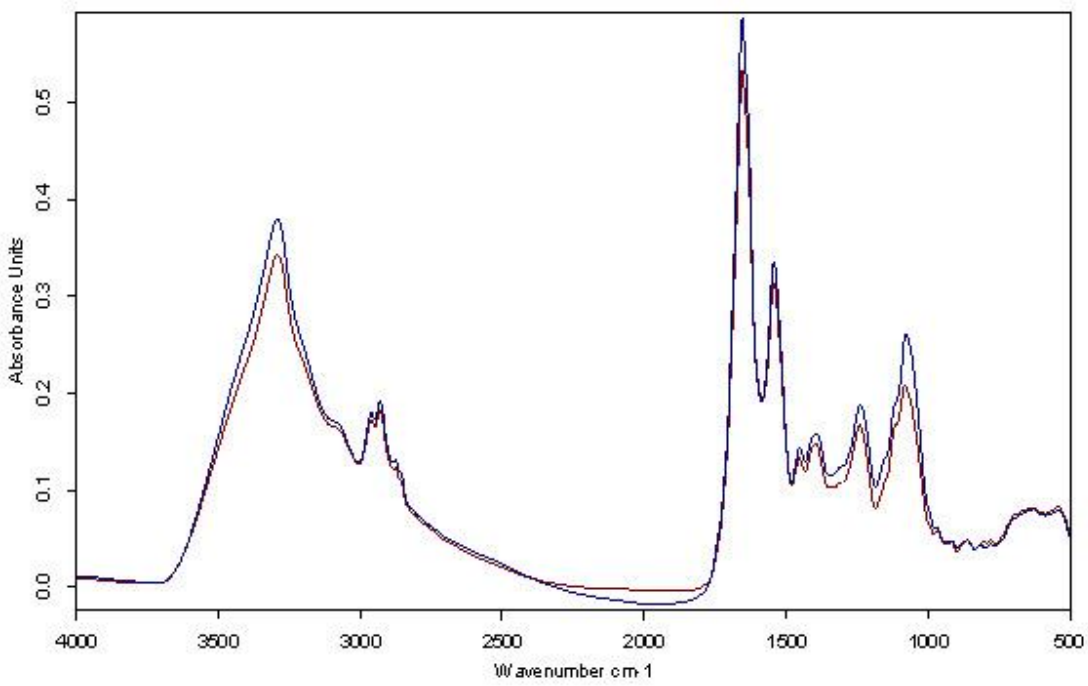

Figure. 3. Average FTIR spectra of *Lb. lactic* and *Lb. bulgaricus*

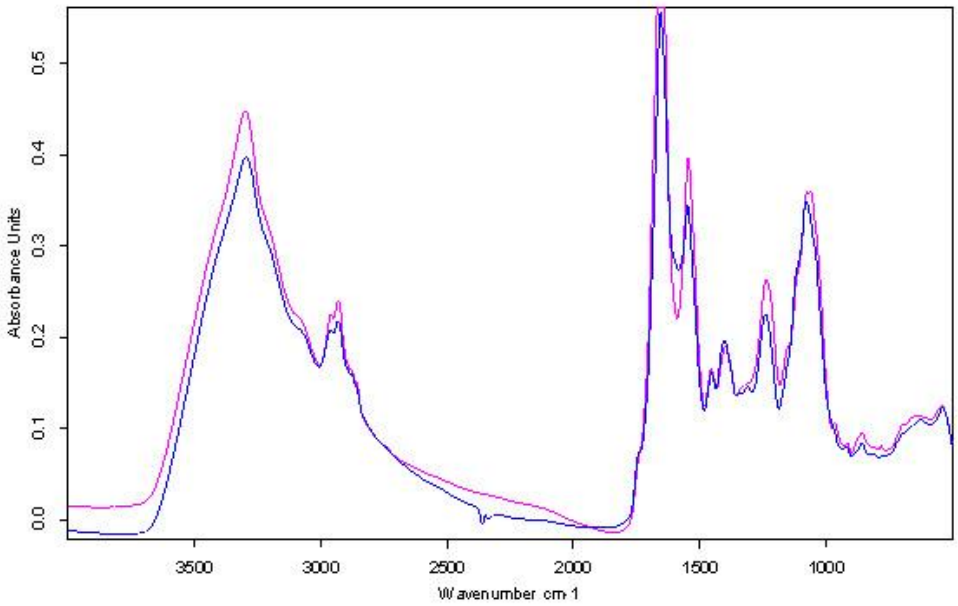

Figure. 4. Average FTIR spectra of *Lb. plantarium* and *Lb. bulgaricus*