

# Effect of Storage's Temperature on Phospholipids Compositions and Viability upon *Lactobacillus plantarum* CWB-B1419

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**Abstract:** *The total lipids of Lactobacillus plantarum CWBI-B1419 were analyzed after freeze-drying. Seven individual lipids classes were identified namely neutral lipids (NL), fatty acids (FA), phospholipids (PL), sterols ester (ES), triglycerides (TG), diglycerides (DG) and monoglycerides (MG). The principal fatty acids identified in most lipid classes were palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). PLs were the major constituents and accounted for 50-60% of the total lipids. PLs were fractionated. PL of Lactobacillus plantarum CWBI-B1419 content phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), sphingomyelin (SM), lysophosphatidylcholine (LPC) and phosphatidylglycerol (PG). It was observed that PG had the highest proportion at most points relative to other PL and was the predominant component of PL (30%-56%). Evolution of individual rate was followed during stored at 20°C and 40°C with or without lithothamne®400, respectively.*

**Keywords:** Lactic acid bacteria, Fatty acids, phospholipids class separation, light scattering detection, survival and temperature.

## 1. Introduction

For the last decade, HPLC has been the method of choice for the separation and quantification of phospholipids (PL) classes. This methodology has the advantage of avoiding the extensive derivatization needed in GC analysis of PL [1]. Previous studies have demonstrated that the properties of an ELSD are useful for a quantitative HPLC detection of lipid classes within different polarity classes [2]. This detection method has been described as highly sensitive for neutral lipids and phospholipids, with an acceptable reproducibility to enable quantification [3] and [4]. A clear benefit of this technique is the possibility of detecting lipophilic substances independent of the absorption properties of eluents or samples and without any probe labeling of detected molecules [5]. The signal intensity of an ELSD seems to be dependent on the development of droplets of eluents by the nebulizer/evaporator, as well as on the detector temperature. Nowadays, the use of an ELSD eliminates problems of derivatization methodology and transparent of mobile phase in UV associated with other HPLC detectors and allows qualitative/quantitative studies of nonvolatile analytes [6]; [7] and [8]. For example, unlike refractive index and low-wavelength UV, ELSD uses multisolvent gradients to improve resolution and perform faster separation of the

eluted compounds [9]-[11]. Therefore, an HPLC-ELSD system could analyze underivatized intact lipids, thus saving time and avoiding losses during derivatization and hydrolysis. Evaporative light scattering is based on the detection of nonvolatile molecules carried by a volatile mobile phase. The column effluents are nebulized to an aerosol, followed by volatile compound vaporization and formation of small solute droplets. The laser light scattered by these droplets is detected by a photodiode [12]. The separation of all PL classes from different matrixes by use of a variety of multisolvent gradients had been reported previously [13]-[16]. Lipids ranging from cholesterol to lysophosphatidylcholine (LPC) can be resolved in a single normal-phase HPLC run using a gradient of increasing polarity. Even nonpolar molecules like hydrophobic pulmonary surfactant proteins can be accurately detected by ELSD [17]. The polarity of different lipid classes constitutes a useful tool allowing PL separation. Solid-phase extraction (SPE) takes advantage of this property and has been used as an alternative to liquid-liquid extraction. A selective elution of the desired compounds can be obtained with SPE by simple fractionation of the analytes through differences in their polarity [18]. For dairy products like freeze-dried LAB, important phospholipids are phosphatidyl-ethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and

phosphatidyl-choline (PC). Important sphingolipids in dairy products are sphingomyelin (SM), glucosylceramide and lactosylceramide. Lysophosphatidylcholine (LPC) and phosphatidic acid (PA) are rarely reported in dairy products; when they do appear, it is likely due to careless sample preparation or to phospholipase activity [19]. The aim of this study was to determine the distribution of individual molecular species of phospho- lipids of *Lactobacillus plantarum* CWBI-B1419 during storage at 20°C and 40°C with or without Lithothamne 400<sup>®</sup> respectively by HPLC-ELSD.

## 2. Materials and methods

### 2.1. Strain and Culture

The fermentation in a 20L bioreactor was performed in triplicate, using the culture medium and the operational parameters of 30°C, 120 rpm, and pH 6, controlled. The fermentation time was 17h. The biomass was centrifuged in a Beckman Avanti J-25 High- Performance Centrifuge Systems (Analys, Belgium) centrifuge. The cream was collected, and analysis of dry matter content, viability, and pH of the cream was performed; this cream was the raw material for the freeze-drying according to [20]. The viable counts were obtained by plate count method after 48 h. Acidification was carried out at 30°C in 150 ml MRS broth inoculated with 1% of 10<sup>7</sup> cfu ml<sup>-1</sup> of the freeze dried sample. The total titrable acidity (% of lactic acid/g DW) was determinate after 18 h, according to the AOAC method, (1997).

### 2.2. Solvents and Standards

**Reagents.** *n*-Hexane, 2-propanol, methanol, and water were HPLC grade from VWR International bvba Haasrode Resaerchpark, Geldenaddksebaan 464 B-3001 Leuven. All other chemicals were analytical reagent grade. L- $\alpha$ -phosphatidylethanolamine (PE), L- $\alpha$ - phosphatidyl DL-glycerol (PG), phosphatidic acid (PA), L- $\alpha$ -phosphatidylcholine (PC), L-  $\alpha$ -lysophosphatidylcholine (LPC), L- $\alpha$ -phosphatidylinositol (PI) and sphingomyelin (SM) with purity of approximately 98% were obtained from Avanti Polar Lipids (Alabaster, USA). To prepare calibration curves, standards were diluted by the appropriate solvent considering its solubility. Duplicate injections of each dilution were used.

### 2.3. Lipid Extraction

Fatty acids were extracted overnight from dried cells of *Lactobacillus plantarum* (2±0.2 g), with ether-ethanol (3:1v/v) mixture according to an adaptation of the method of Ito et al., (1969). The extraction was performed by successively adding 10mL of chloroform containing butyl-hydroxy-toluene (0.01%), 10 mL of chloroform and 12.8 mL of NaCl (1.56%). After each new addition, the mixture was homogenized by a 20s blending period (Ultra-turrax, IKA, Staufen, Germany). The mixture was centrifuged for 10 min at 6000 g. Total lipids (TL) were obtained by collecting an aliquot (10 mL) of the chloroform phase. During the extraction procedure, tubes were kept at 0-4°C.

### 2.4. Solid-Phase Extraction (SPE)

After evaporation of the chloroform, the TL extract was diluted in 1.5mL of chloroform/methanol (2:1, vol/vol). The organic phase was collected into a conical vial, evaporated to dryness under a nitrogen stream, and made up to 0.5-mL final volume with chloroform. The extract was applied to the top of a normal-phase silica cartridge (Bond Elut NH<sub>2</sub>, Anion Exchange, varian Inc. N.V, Varian S.A, Belgium) that had been previously conditioned with 10 mL of chloroform. Sequential elution was performed with the cartridge column was equilibrated by rinsing twice with 2 ml of hexane using the Supelco extraction apparatus. Lipids dissolved in 10 mL of chloroform were loaded onto the column and the chloroform was pulled through. Thereafter, the column was eluted with 4 ml of a chloroform-2-propanol 2:1 mixture (neutral lipid fraction). Then, 4 ml of ether containing 2% acetic acid followed by 4 ml of methanol was applied to elute free fatty acids and neutral phospholipids, respectively [21]. Finally, acidic lipids were eluted using 4 ml of a mixture of hexane-2-propanoethanol-0.1 M ammonium acetate in water-formic acid 420:350:100:50:0.5 containing 5% phosphoric acid 20 mL of chloroform, 5 mL of acetone, and 20 mL of methanol. To prevent the oxidation of polyunsaturated chains during PL extraction, 0.02% wt/vol BHT was added to the solvents. This procedure separates neutral lipids, and polar lipids [22] and [23]. The methanol extract was dried at 40°C under a nitrogen stream, dissolved in 1 mL of chloroform, and filtered through a 0.2  $\mu$ m nylon membrane, the PL fraction was diluted in 2mL *n*-Chloroform–methanol solution (80:20 before injection onto the HPLC according to **table 1**.

**Table 1:** HPLC- Gradient scheme

	Time (min)									
	0	5	10	13	13:01	15	17	24	25	30
MeOH (%)	0	0	45	45	60	80	100	0		
ACN (%)	0	0	0	0	0	0	0	100	100	
Isopropanol/ Hexane (40:60, v/v)(%)	100	0	0	0	0	0	0	0	0	100
Isopropanol/ Hexane/ H <sub>2</sub> O (63:35:2, by vol) (%)	0	100	55	55	40	20	0	0	0	0
Flow (mL/min)	1	:	0.8	0.8	1	1	1	0.8	0.8	0.8
Percentage of each component of the mbile phase is indicated ACN acetone										

### 2.5. HPLC-ELSD of the PL fraction

**HPLC methodology:** The Spectra System HPLC from Thermo Separation Products (TSP) included a P4000

quaternary pump and an autosampler AS3000 with an injection valve of 100- $\mu$ L sample loop. The separation system consisted of a 3  $\mu$ m Waters Spherisorb Silica column of 120  $\times$  4.6 mm with a silica guard column. The column

oven was set at 30°C. All equipment was connected through an SN4000 interface with the data acquisition system software PC1000 (TSP).

**Detector calibration.** The evaporative light-scattering system consisted of a ELSD Model 500 detector (Alltech, Deerfield, IL). An interface module converted the ELSD analog signal to digital data that could be processed by the computer. The suitable signal-to-noise ratio was determined by injection and detection of PL standards. A drift tube temperature of 70°C, a gas flow rate of 1.98 standard liters per minute, and a gas pressure of 13.1 psi (0.090 MPa), were the most convenient parameters. High-purity N<sub>2</sub> was used as nebulizer gas. Under these conditions, vaporization of the solute did not occur, and there was also a stable baseline with attenuation factor of 1. The PL fractions (10 µL) were injected. The gradient system used for the separation of the major PL classes is given in Table 1. All solvents were degassed and filtered prior to analysis.

**Repeatability and recovery assays.** Repeatability was determined for all the analytes, which were present in total

lipids. One batch sample was divided into three aliquots of 1 mL each, which were subjected to the HPLC-SPE procedure. The percent recovery was determined for PC, the component present in the largest amount in the analyzed sample (74-85% of the total PL). Total lipid (1 mL) was enriched with 2 mg of standard and analyzed by using the HPLC-SPE procedure. Three independent samples were prepared.

**Calibration curves.** A response calibration curve for each class of PL was prepared. Standards were diluted with the appropriate solvent, considering the solubility of the PL. Duplicate 10-µL injections of each dilution were used in the range from 1 to 40 µg for three separate replicates for each analyze. The hypothesis of linearity was tested by using the Linear Regression Model (GLM) procedure, a package program of the Statistical Analysis System (SAS, Cary, NC).

### 3. Results

#### 3.1. *Lb. plantarum* CWBI-B1419 growth characteristics

**Table 2:** Survival after freeze-drying process at 20°C and 40°C for 90 days, Kinetic parameters of the growth and substrate composition of *Lb. plantarum* CWBI-B1419 in batch culture

		Cell count (cfu/DW)				Parameters <sup>b</sup>			
	Before F.D	During storage		% Survival		Growth	µ (d <sup>-1</sup> )	X <sub>max</sub> (g/l)	Y <sub>x/sub</sub> (g/g)
		20°C	40°C	20°C	40°C				
C	8,8.10 <sup>11</sup>	1,5.10 <sup>11</sup>	1,7.10 <sup>10</sup>	17	2				
CL	7,1.10 <sup>11</sup>	2,7.10 <sup>11</sup>	4,9.10 <sup>10</sup>	38	7		0.59 ± 0,01	10.2 ± 0,02	0.48 ± 0,02

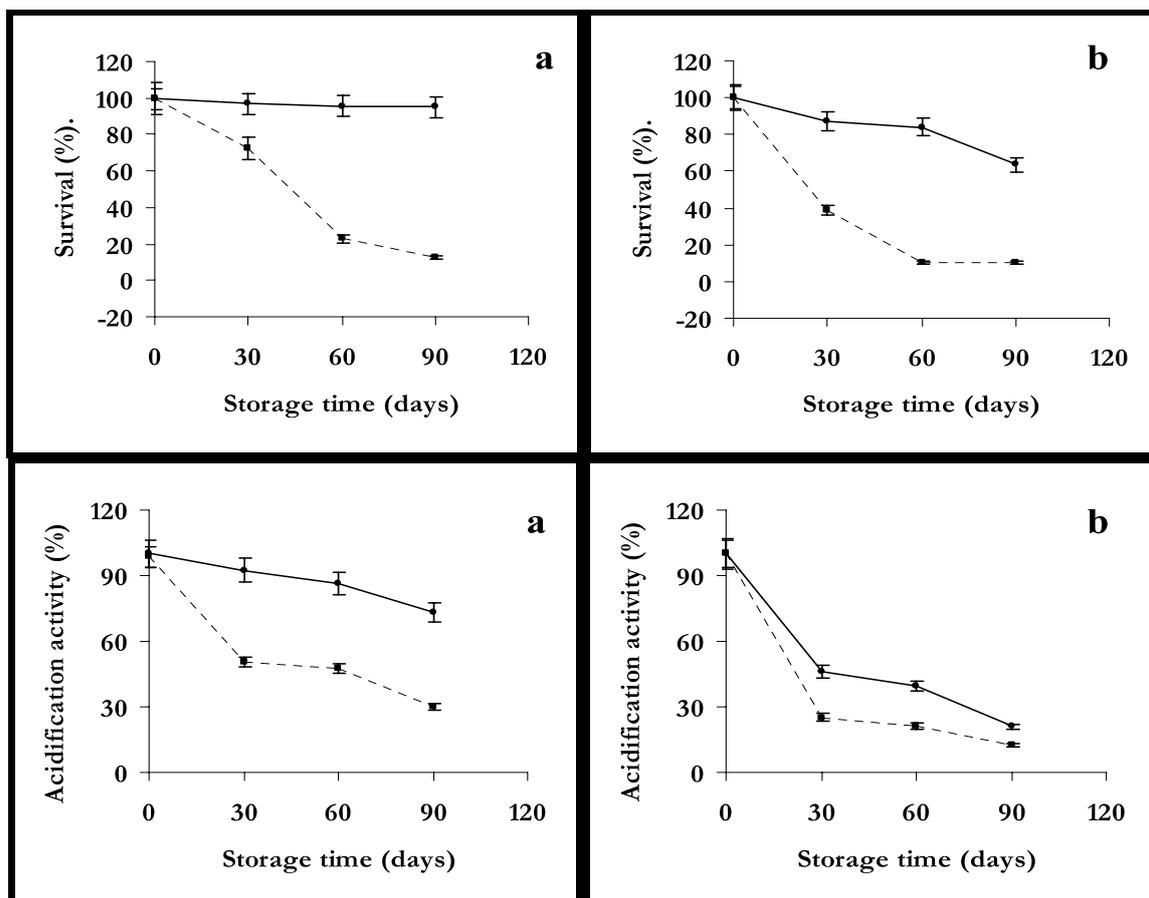
<sup>a</sup>All values were means of three measurements and expressed as means ± standard deviation.

<sup>b</sup>µ, specific growth rate (d<sup>-1</sup>); X<sub>max</sub>, maximum biomass concentration (g/l); Y<sub>x/sub</sub>, growth yield based on substrate (g/g). C: cells without Lithothamne, CL: cells with Lithothamne.

The kinetics of cell growth and glucose consumption of *Lb. plantarum* are shown in table 2. The bacteria grew well with specific growth rate of 0.59d<sup>-1</sup>. The growth yield coefficient, based on substrate, Y<sub>x/sub</sub>, was 0.48 g/g. The highest biomass concentration, X<sub>max</sub> of 10.20 g/l was obtained after 16 hours. The kinetic growth parameters obtained in this study were comparable to those reported by [20].

#### 3.2. Biochemical behavior of *Lb. plantarum* CWBI-B1419 during storage

*Lb. plantarum* CWBI-B1419 was produced in the bioreactor, freeze-dried, and held at 20°C or 40°C in vacuum-sealed aluminum foil for 90 days.



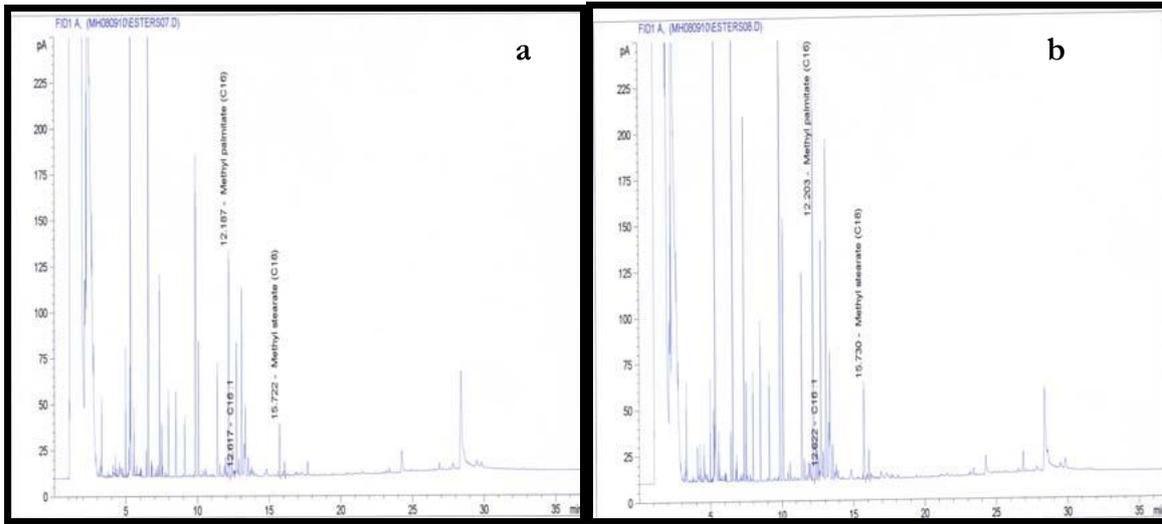
**Figure 1.** Behavior of freeze-dried *Lb. plantarum* CWBI-B1419 in function of survival rate and acidification activity during 90 days at : **a)** 20°C or **b)** 40°C, with Lithothamne (-○-) or without Lithothamne (-■-). Values as means ± standard deviation (SD,  $n = 3$ )

According to the curves illustrated in **figure 3**, the decreasing of acidification activity and survival rate are closely related to the storage temperature. After 90 days of storage, there is a survival and acidification activity significantly higher for lyophilized powder stored at 20°C vacuum foil contrast to those stored at 40°C in the same conditions. For comparison, the viable population of the strain lyophilized and stored in sealed foil under vacuum has been affected and we note that from an initial population of  $8,8 \cdot 10^{11}$  to  $1,5 \cdot 10^{11}$  or  $1,7 \cdot 10^{10}$  cfu/g without Lithothamne, we have a survival percentage of 17% and 2% respectively after 90 days at 20°C and 40°C. But after 90 days at 40°C under the same conditions but with Lithothamne, the viable population was about  $7,1 \cdot 10^{11}$  to  $2,7 \cdot 10^{11}$  or  $4,9 \cdot 10^{10}$  cfu/g with a survival of 38% and 7% at 20°C and 40°C

respectively. When the activity of acidification strain lyophilized and stored under the conditions mentioned above there is a decrease of 24% or 60% with freeze-dried cell with Lithothamne after a period of 90 days storage at 20°C and 40°C, respectively.

### 3.3. Free fatty acids and lipids compositions

Free fatty acids (FFAs) from two freeze-dried powders of *Lactobacillus plantarum* CWBI-B1419 (with Lithothamne and without Lithothamne at 20°C and 40°C, respectively) were analyzed. **Figure 4** shows the mean relative contents of these FFAs.



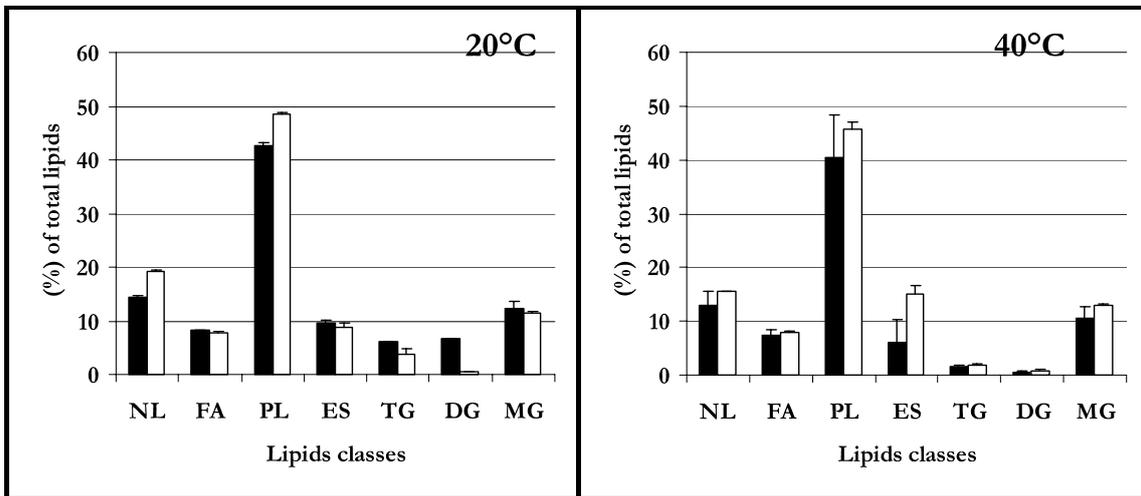
**Figure 2:** Free fatty acids profile of *Lb. plantarum* CWBI-1419 **a)** after freeze-drying without Lithothamne, (C); **b)** after freeze-drying with Lithothamne, (CL).

**Figure 34.** Free fatty acids profile of *Lactobacillus plantarum* CWBI-B1419 **a)** after freeze-drying

without lithothamne®400, (C); **b)** after freeze-drying with lithothamne®400, (CL). Assay of the constituent fatty acids of *Lb. plantarum* CWBI-B1419 revealed predominantly esters of palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids in variable proportions. The palmitic (C16:0) accounted from 52.4% to 55.4% without or with Lithothamne at the end of freeze-drying, respectively. Palmitoleic (C16:1) making a smaller (on average 7.0-7.4%) and oleic (C18:1) from 23.5% to 27.2%, without or with Lithothamne, respectively. The C18:2 and C18:3 represent the smallest percentages less than 1% to the total fatty acids. There was no significant difference in FFAs of C and CL.

**3.4. Fatty acids distribution in lipids classes**

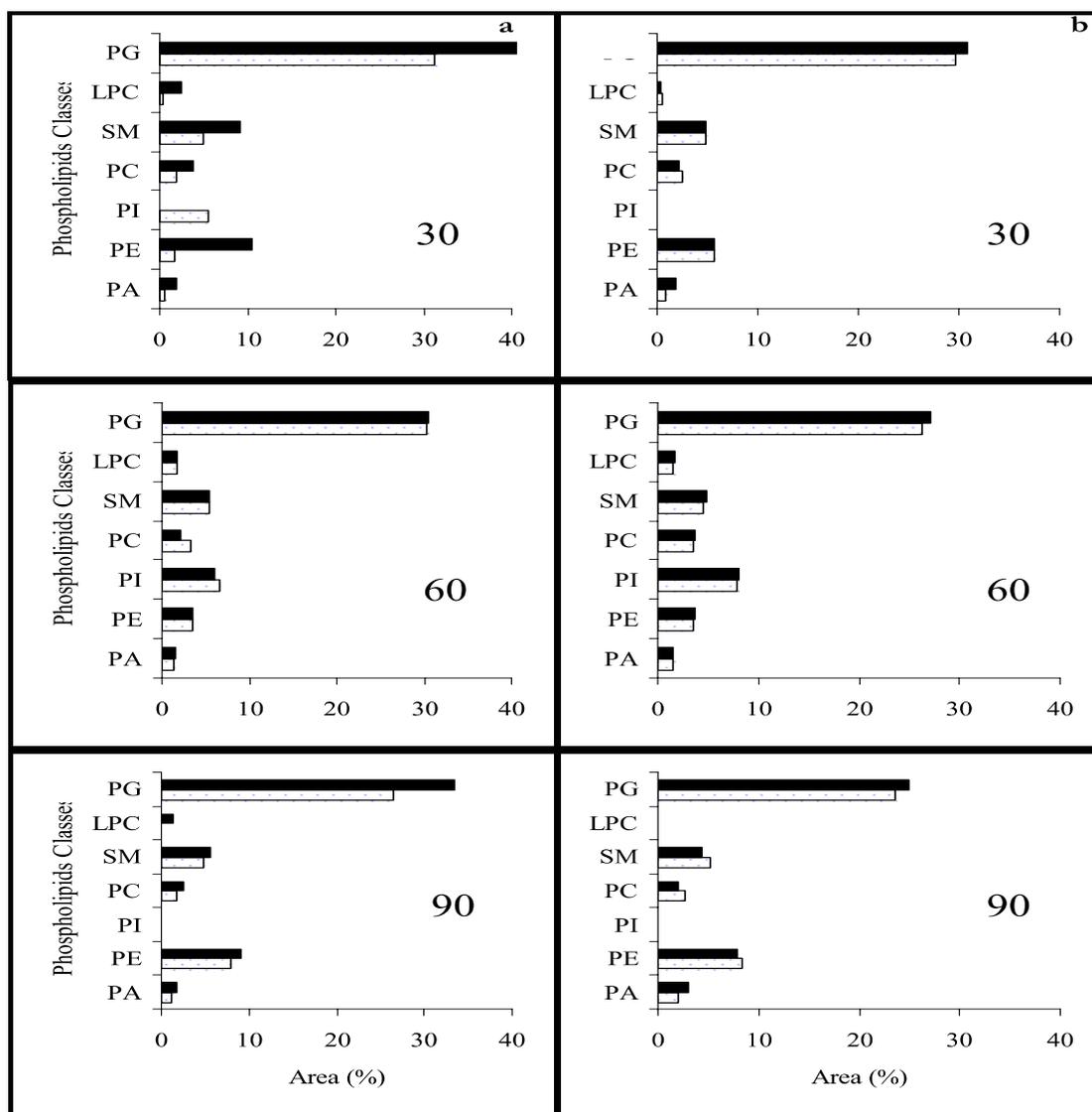
Fatty acid profiles of different lipid fractions of *Lactobacillus plantarum* CWBI-B1419 are depicted in **figure 3**. PL was mainly composed of unsaturated fatty acids, C16:1 and C18:1, which altogether represented 60.54% of the total fatty acids (TFA) in this lipid fraction. The saturated fatty acids palmitoleic C16:0 and stearic C18:0 accounted 17.8% of the total. PL content high proportion of saturated fatty acids than NL. There was significant difference in NL and PL according to saturated fatty acids. The fatty acid profile of PL was quite different from those of NL and TL.



**Figure 3.** Individual lipid class content (% of total lipids) of *Lb. plantarum* CWBI-B1419: **a)** □ freeze-dried strain without Lithothamne, **b)** ■ freeze-dried strain with 8% Lithothamne. Values are represented as means ± standard deviation of triplicates.

The contents of other MUFAs, such as C18:1 and C16:1 was also in the sample order but slightly high in PL fraction. The percentages of unsaturated fatty acids of PL were 8.99% and 31.5% for C16:1 and C18:1 respectively (data are not show).

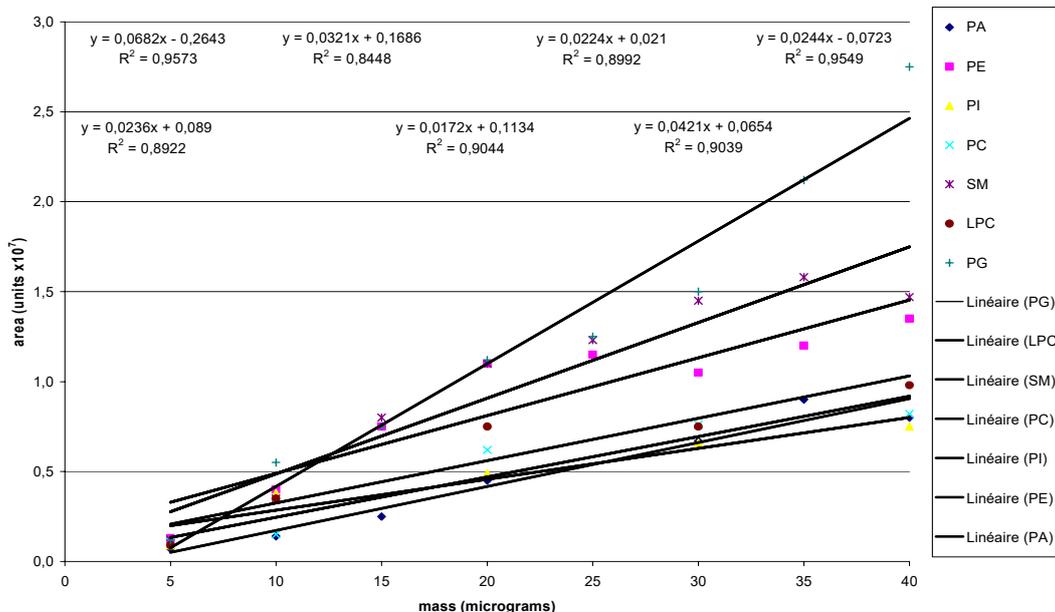
3.5. HPLC procedure for the separation and quantification of phospholipids in the PL fraction



**Figure 4:** Percentage composition of phospholipids classes in freeze-dried powder of *Lb. plantarum* CWBI-B1419. Symbols: ■ freeze-dried powder with Lithothamne (CL), □ freeze-dried powder without Lithothamne (C) at 30, 60 and 90 days.

The detection methods of PL in HPLC involve direct measurement of total phospholipids. Direct measurement of PL has been accomplished using an automated total phospholipids analyzer named Evaporative Light Scanning Detector (ELSD). Coupled with HPLC system. It was found that fatty acid of *Lb. plantarum* CWBI-B1419 was dominated by NL and PL which represented almost 20% and 50% of total lipid extract (**figure 3**). As a consequence, quantification of the polar lipid classes required purification of the total lipid extract with aminopropyl cartridges by SPE. After SPE, the main rapeseed lipid components PA, PE, PI, PC, SM and LPC were separated with symmetrical peaks as shown in **figures 3 & 4**. PL from power stored at 20°C has

been described as having the following phospholipids distribution after 90 days: PA (7.45, 5.31 wt%); PE (21.15, 19.98 wt%); PI (34.26, 31.46 wt%); PC (7.05, 6.0 wt%); SM (22.87, 22.27wt%) and LPC (18.10, 10.09%wt) respectively, with or without Lithothamne®. The profile at 40°C with or without Lithothamne was similar. Linearity was obtained within the 5 to 40 µg range ( $p < 0.001$ ) by using the Linear Regression Model (GLM) procedure, a package program of the Statistical Analysis System (SAS, Cary, NC). Each point represents the mean value of duplicate injections for three separate replicates.



**Figure 5:** Calibration curves mass of PL vs ELSD response in 10 µl of injection volume.

**Figure 37.** Calibration curves: mass of PL vs. ELSD response in 10 µL of injection volume. Results are given as linear curves of phospholipids extracted of three separate extractions from the same sample of *Lb. plantarum* CWBI-B1419 freeze-dried. Each curve includes a linear equation of the regression line as well as the  $R^2$ .

#### Analyse des phospholipides de *Lactobacillus plantarum* CWBI-B1419

The experiments were performed thrice. SPE, the solid phase extraction has yielded the following phospholipids: PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin; LPC lysophosphatidylcholine; PG, phosphoglycerol as shown by **figure 5**.

## 4. Discussion

Detection of phospholipids is favored because (i) lipids are extracted by the hydrophobic solvent and (ii) SPE selectively desorbs surface-active molecules such as polar lipids, though not all types equally well. Many studies have focused on the lipid composition of *Lactobacillus* spp. [24]-[26]. Authors such as [27], [28] showed that lactobacilli are generally composed of n-C16:0, n-C18:1 and C19 CYC-carboxylate as the main constituents with a small amount of n-C14:0, n-C16:1, and n-C18:0 and traces in some species [29]. These atypical profiles in terms of fatty acids are those of gram-positive, but the main groups are the common gram-positive bacteria [30]. The analysis of polar groups in *Lb. plantarum* B1419-CWBI revealed the same trends and showed that the majority of polar lipids is composed of phosphatidylglycerol (PG). These results are fully consistent with studies by [31]. As for other components such as phosphatidic acids, diphosphatidylglycerol (cardiolipin), phosphatidylglycerol [32], [33] phosphoglycerolipids and diglycosyldiacylglycerol [34] there are small quantities. During storage we noticed a decrease in PG for freeze-dried at 20°C while at 40°C not only PG but also decreased PE and SM begin a significant decline in 90 days. These results are similar to those we obtained with *Lb. plantarum*. At this temperature the powders without Lithothamne are most vulnerable. As explanation and following this period the decrease in the ratio unsaturated/saturated fatty acids and cell death

occurred essentially at the same rate (data are not showed). As stated before, the mechanisms of death during storage are still unknown but from these results it seems evident that lipid oxidation and survival during storage may be related. This is supported by indirect evidence presented in previous reports. For example, it is reported that the presence of antioxidants increased the survival rate of dried bacteria during storage [35], [36]. [37] found a strong similarity between the loss of viability and the increase in free-radical concentration during storage of freeze-dried *Serratia marcescens*. Work done by [38] supports the hypothesis that reactions between carbonyl compounds and cellular components are a major cause of mortality during storage of dried microorganisms. The nature of these compounds was not presented but it is possible that they are products formed during lipid oxidation. [39] reported that, in eukaryotic cells, extensive lipid peroxidation can result in membrane disorganization by peroxidizing mainly the highly unsaturated fatty acids leading to changes in the ratio of unsaturated to other fatty acids. The uncontrolled peroxidation of biomembranes can thus lead to profound effects on membrane structure and function, and may be sufficient to cause cell death.

## 5. Conclusion

The present method thus *Lactobacillus* phospholipids almost free of triglycerides in one step and does not require the pre-extraction of total lipids is conventional. These methods have been successfully used and have given a satisfaction in our analysis for our isolation of phospholipids from bacteria and plant tissues. Work for monitoring changes in phospholipids classes will be conducted for other species in order to know the limits of the method.

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