

**DETECCIÓN DE *Lactobacillus plantarum* 299V USANDO BIOSENSORES BASADOS EN MICROCANTILEVERS CON MICROSCOPIA DE FUERZA DINÁMICA****DETECTION OF *Lactobacillus plantarum* 299V USING MICROCANTILEVER-BASED BIOSENSOR WITH DYNAMIC FORCE MICROSCOPY**

A.G. Mendoza-Madrigal¹, J.J. Chanona-Pérez^{1*}, J.V. Méndez-Méndez², E. Palacios-González³, G. Calderón-Domínguez¹ y H. Hernández-Sánchez¹

¹Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Plan de Ayala y Carpio s/n, Col. Santo Tomás, C.P. 11340, México, D.F.

²Centro de Nanociencias y Micro y Nanotecnologías, Instituto Politécnico Nacional, Luis Enrique Erro s/n, Unidad Profesional Adolfo López Mateos, Col. Zacatenco, C.P. 07738, México, D.F.

³Laboratorio de Microscopía de Ultra Alta Resolución, Instituto Mexicano del Petróleo, Eje Central Lázaro Cárdenas 152, Col. San Bartolo Atepehuacan, C.P. 07730, México.

Recibido 19 de junio de 2013; Aceptado 1 de julio de 2013

Abstract

The aim of this study was the detection of active *L. plantarum* 299v (probiotic microorganism) growth using microcantilever based biosensors in air and in dynamic mode by atomic force microscopy. Commercial cantilevers were cleaned with Piranha solution in order to eliminate contaminants and were functionalized with silylating solution; afterwards the cantilevers were coated by an agarose layer using the capillaries technique. An atomic force microscope in tapping mode was required to evaluate the resonance frequency shift of commercial cantilevers inoculated with *L. plantarum* 299v. Humidity and temperature were controlled inside an atmospheric hood during the biodetection. The resonance frequency curves were seen to be narrower with higher Q factor values (~219). The results showed that the resonance frequency shifted by approximately 5.2±0.8 kHz on the inoculated cantilevers throughout the growth kinetics. From the resonance frequency curves and known mechanical properties of the cantilevers, the biosensor sensitivity was determined to be 383±3 pg/Hz and the biosensor can detect ~400 bacteria. In addition, *L. plantarum* growth on the cantilever's surface was confirmed by scanning electron microscopy. The results showed that it is possible to construct a microbiosensor by using commercial cantilevers and atomic force microscopy. These sensors can be used as a platform for the detection of microorganisms associated with functional foods.

Keywords: biosensors, cantilever, atomic force microscopy, foods.

Resumen

El objetivo de este estudio fue la detección del crecimiento activo de *L. plantarum* 299v (microorganismo probiótico) utilizando microcantilevers como biosensores en modo dinámico en microscopía de fuerza atómica (MFA). Cantilevers comerciales se limpiaron con solución Piraña y fueron funcionalizados con solución sililada; posteriormente se recubrieron con agarosa utilizando la técnica de capilares. Un MFA en modo intermitente se utilizó para evaluar el cambio de la frecuencia de resonancia de cantilevers comerciales inoculados con *L. plantarum* 299v. La humedad y la temperatura se controlaron dentro de una campana atmosférica durante la biodetección. Las curvas de frecuencia de resonancia mostraron valores altos de factor Q (~219). Los resultados mostraron que el desplazamiento de la frecuencia de resonancia cambia 5.2 ± 0.8 kHz en cantilevers inoculados a lo largo de la cinética de crecimiento. A partir de las curvas de frecuencia de resonancia y las propiedades mecánicas de los cantilevers, la sensibilidad del biosensor fue 383±3 pg/Hz y el biosensor puede detectar ~400 bacterias. El crecimiento de *L. plantarum* en los cantilevers se confirmó por microscopía electrónica de barrido. Los resultados mostraron que es posible la construcción de microbiosensores mediante el uso de cantilevers y MFA como plataforma para detección de microorganismos asociados a alimentos funcionales.

Palabras clave: biosensores, cantilever, microscopía de fuerza atómica, alimentos.

*Autor para la correspondencia. E-mail: jorge.chanona@hotmail.com

1 Introduction

Lactobacillus plantarum is a member of the human microbiota with a strong ability to preserve food and prevent spoilage. *L. plantarum* has been successfully tested for its capability to adhere to human colonic cell lines to survive gastrointestinal passage (Dhanani *et al.*, 2001). *L. plantarum* has been largely used as a culture starter in the food industry and for the development of probiotic foods (Laparra and Sanz, 2009). During manufacturing of probiotic foods, it is usually required as a routine proof of the counting of viable probiotic bacteria. The analysis of this can take at least 24 h, which implicates a significant economic cost. Therefore, the food industry requires faster and more sensitive methods to evaluate active bacterial growth. Biosensors could be used as a rapid detection method, taking advantage of the adhesive properties of *L. plantarum* (Dhanani *et al.*, 2011), it can be studied on biological surfaces by using cantilevers as biosensor for *in situ* detection of probiotic microorganisms. The main advantages of biosensors over traditional analytical techniques for the detection of food microorganisms are their cost-effectiveness, fast and portable detection, which makes possible both *in situ* and real time monitoring without extensive sample preparation (Van Dorst *et al.*, 2010). Currently, biosensors have seen a plethora of applications in biological monitoring, environmental sensing, food safety, medicine, security and defense (Lazcka, 2007; Su *et al.*, 2011; Vo-Dinh *et al.*, 2001).

Biosensors are integrated devices based on receptor-transducer elements, which are able to provide selective quantitative or semi-quantitative analytical information from diverse signals types (electrical, mechanical, magnetic, chemical, thermic, etc). Biosensors also require a biological recognition element such as organic thin films, tissues, microorganisms, organelle, cells, enzyme, antibody, proteins, nucleic acid, biomimetic molecules, etc., as well as a suitable signal transduction method (generally optical, piezoelectric or electrochemical) in such a way that the binding or reaction between the target and the recognition element is translated into a meaningful signal (Thévenot *et al.*, 2001; Velusamy *et al.*, 2010).

Micromechanical bending and oscillating cantilevers have been used like a new class of biological and chemical sensors. The elegance of these sensing methods is that the detection of an analyte requires no labelling and the biosensor designs only differ in the functional layers deposited on the

cantilever surface (Berquand *et al.*, 2005). Atomic force microscopy (AFM) provides an approach to create and evaluate microbiosensors; the use of cantilevers as force sensors in AFM is a sensing platform which offers excellent sensitivity and a very low detection limit. Cantilevers are of particular interest because are easy to fabricate, can perform rapid real-time measurements and can be miniaturized for incorporation into lab-on-a-chip applications (Waggoner and Craighead, 2007). AFM has been used to measure the specific binding forces of individual ligand-receptor complexes or microorganism growth. This involves attaching specific biomolecules or chemical compounds onto AFM cantilevers and recording force-distance as well as dynamic response curves of functionalized surfaces (Raiteri *et al.*, 2001; Su *et al.*, 2011). Recently, numerous cantilever-based sensors have been reported. These include cantilevers for the detection of a DNA sequence specific to the protein of interest (Ilic *et al.*, 2005; Savran *et al.*, 2004), specific detection of streptavidin (Shu *et al.*, 2007), antibodies used to bind the protein of interest (Yen *et al.*, 2009), viral detection (Gunter *et al.*, 2003) and microorganisms such as *Escherichia coli* O157:H7 (Campbell and Mutharasan, 2005) and *Vibrio cholerae* O1 (Sungkanak *et al.*, 2010).

In dynamic mode, a cantilever is driven at its resonance frequency f_0 by a piezoelectric actuator. If target molecules are adsorbed onto the cantilever, its resonance frequency will decrease by Δf due to mass loading (Lang *et al.*, 2002) This frequency shift is proportional to the mass added (Nugaeva *et al.*, 2005). This operation principle of the cantilevers has been taken advantage of for the detection of pathogens, microorganism and biomolecular interactions. In dynamic mode, a cantilever resonator can be treated as a harmonic oscillator with dominant modes of vibration dependent only on the spring constant (k) and the effective mass (m), (Sungkanak *et al.*, 2010). According to Ricciardi, *et al.* (2001), if a substance is immobilized or when microorganisms grow on the cantilever surface, the variation of resonant frequency can be directly correlated with the added mass (Δm):

$$\Delta m = \frac{\Delta f}{f_0} m \quad (1)$$

Here f_0 is the resonance frequency before the binding of molecules or growth of microorganisms. This equation has been used effectively for cantilevers used as biosensors in several biological systems such as proteins, cells and antibodies among others.

Other important issue is the cantilevers signal

sensitivity; for its evaluation usually the quality factor (Q) or Q -factor is used (Minary-Jolandan *et al.*, 2012) which can be calculated by means of the following expression (Giessibl, 2003; Lübbe *et al.*, 2010; Minary-Jolandan *et al.*, 2012):

$$A = \frac{A_{max}}{1 + \left(\frac{f-f_0}{c}\right)^2} \quad (2)$$

Here, c is half the full-width at half maximum of the resonance frequency peak (Δf_{whm}), f_0 is the resonant frequency, while Q is the quality factor defined as $Q = f_0(\Delta f_{whm})^{-1}$ and A_{max} is the maximum amplitude at f_0 (Johnson and Mutharasan 2012).

Cantilevers with high Q -values improve the force sensitivity in non-contact mode on AFM. Typically the Q -factor can be evaluated from curve of a frequency-amplitude plot by fitting with a Lorentzian function of a single harmonic oscillator (Minary-Jolandan *et al.*, 2012; Lübbe *et al.*, 2010; Seo and Jhe, 2008). High Q -values can be associated with narrow resonance peak of the AFM response curve and a high sensitivity (Lübbe *et al.*, 2010; Ricciardi *et al.*, 2010). Although several biosensors based on microcantilevers have been developed, these have been mainly for the detection of pathogen microorganisms. The development of biosensors for monitoring probiotic microorganisms in food industry is still scarce. For this reason the aim of this study was the detection of active *L. plantarum* 299v (probiotic microorganism) growth, using microcantilever based biosensors in air with dynamic mode AFM.

2 Materials and methods

2.1 Strain and culture method

L. plantarum 299v was obtained from the culture collections of the food science department (Escuela Nacional de Ciencias Biológicas). The strains were grown in liquid Man Rogosa Sharpe (MRS) broth (Charalampopoulos *et al.*, 2009; Zago *et al.*, 2011). For the preparation of the inoculums, the microorganisms were obtained after inoculating 1 colony of *L. plantarum* from a Petri plate, into a flask containing 50 mL of MRS broth. This mixture was left overnight at 37°C and 180 rpm in an orbital shaking incubator (ESEVE, INO-65OV-7, Mexico). Bacteria from 1 mL were harvested by centrifugation (Beckman, J2-MC, USA) and the resulting pellet was suspended in 9 mL of MRS broth. All the treatments

had an initial concentration of *L. plantarum* of 1×10^3 CFU/mL which was verified by pour plate technique.

2.2 Cantilever functionalization

Rectangular cantilevers 125 μm long, 50 μm wide and 5 μm thick with a spring constant of 80 N/m (MPP-11100-10 model, Bruker, USA) were used as the microbiosensors in this study due to the commercial feasibility, geometric and mechanic characteristics which are appropriate for bacterial detection. To remove surface contaminations, the cantilevers were cleaned for 20 min in "Piranha" solution (95-97% H_2SO_4 concentration in 30% H_2O_2 , 1:1) and rinsed twice with sterile-filtrated water and once with deionized water. This cleaning procedure was performed twice. Cantilever functionalization was performed following the methodology proposed by Gfeller *et al.* (2005), that consists of immersing the cantilevers in silylating solution at 1% (3-glycidyloxypropyl)-trimethoxysilane and 0.5% N-ethyl-diisopropylamine (Sigma Aldrich) in water, for about 4 h at room temperature to form a self-assembled monolayer. This provided a hydroxyl surface reactive to primary hydroxyl groups (e.g., agarose). After the silylation, the cantilevers were rinsed twice with toluene for 20 min and dried under an argon atmosphere.

In order to verify the functionalization process, the cantilever's surfaces were analyzed before and after treatment with "Piranha" solution, and as well as after the silylation treatment by means of X-Ray Photoelectron Spectroscopy (XPS, K-Alpha Thermo Scientific, UK). General spectra were obtained from each cantilever with the method of Brundle *et al.* (2010).

Agarose (A9414-5G, Sigma-Aldrich) was dissolved in deionized water at a concentration of 0.8% (w/v), melted and stored at 60°C. Just prior to use, the pH of the agarose solution was adjusted to pH 11 with 5M NaOH. The agarose coating of each cantilever was accomplished with a micromanipulation system (Figure 1A-B). Cantilever chips were placed onto a microscope slide in order to position them with an optical microscope stage with clips (BX51, Olympus Japan). An acrylic plate 7 cm wide and 12 cm long was used to place a syringe where commercial capillaries with an inner diameter about 0.40 mm (Leuka, Mexico) were put up at the end of the syringe, this plate was put into another optic microscope stage (BX51, Olympus Japan).

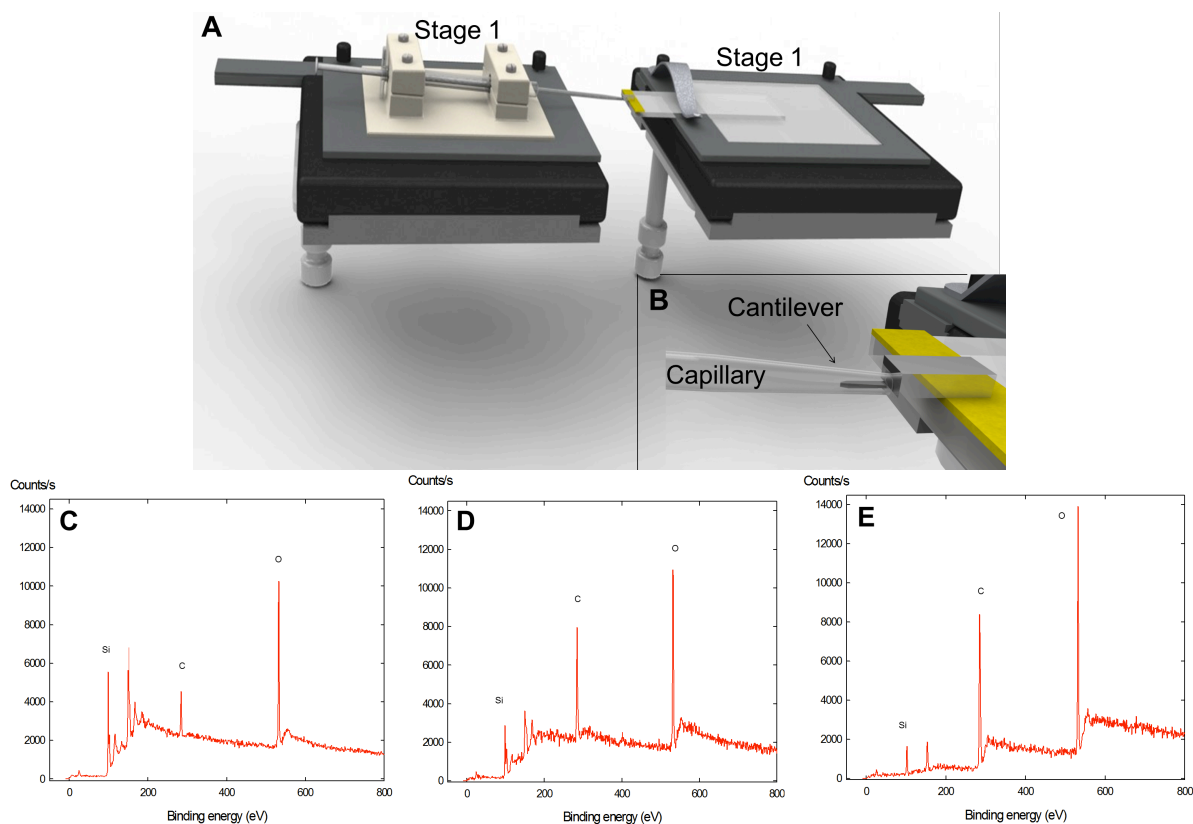


Fig. 1. Cantilevers functionalization steps: (A) Schematic diagram of micromanipulation system, (B) magnification of cantilevers functionalization. XPS general spectra of cantilevers: (C) before cleaning, (D) after cleaning, (E) after silylanization.

The stage with the cantilever was positioned until it reached the inside of the capillary and the cantilever remained there for about 10 s (Figure 1B). Subsequently, the functionalized cantilevers with agarose were stored in a desiccator for 2 days ahead of their use in growth experiments. The agarose functionalization was verified by confocal laser scanning microscopy (LSM710 Carl Zeiss, Germany) taking advantage of the autofluorescence of the silicon and agarose layer. The samples were excited with four laser lines (405, 488, 555 and 639 nm).

2.3 Biodetection experiments

The active growth evaluation of *L. plantarum* was performed with an atomic force microscope diMultiMode V (Veeco, USA) with NanoScope V control hardware (Digital Instruments, USA) in dynamic mode. The measurement principle

consists of a laser focused onto the end of an oscillating cantilever. The reflected laser beam is then deflected onto a position-sensitive detector (PSD), where resonance frequency and amplitude can be measured. For all the measurements, the cantilever coated with agarose was immersed for 15 min in a suspension with *L. plantarum* at a concentration of 10^3 CFU/mL and liquid specific *Lactobacillus* medium MRS (Charalampopoulos *et al.*, 2009) in the aforementioned capillaries; afterwards they were used for biodetection experiments. Is it important to mention that during cantilever incubation some amount of nutrients of the growth medium could be adsorbed on the cantilever surface, thereby, these nutritive compounds can be used by microorganism for its growth during biodetection experiments. For the detection of *L. plantarum*, the cantilevers previously inoculated were mounted in the cantilever holder for 4 h and resonance frequency-amplitude curves taken every 30 min. Functionalized cantilevers with

an agarose film without been inoculated with *L. plantarum* were used as reference cantilever. In all experiments, to maintain the temperature and relative humidity, the AFM head was placed inside an atmospheric hood (Veeco, USA). The method used for control temperature and relative humidity was similar to that reported by Zhu *et al.*, (2007) with minor modifications. The relative humidity was achieved and maintained with a humidifier (Vitalys Plus, VUH-5, China) connected to an atmospheric hood. During the experiments the temperature and relative humidity remained around 22 ± 2 °C and 90 ± 2 % RH, which were monitored inside of atmospheric hood with a digital termohygrometer (Acurite 00611, Chaney Instruments Co., USA, accuracy of ± 1 °C and ± 1 % RH). Every experiment was performed 3 times in order to ensure reasonable reproducibility. Finally, in order to verify *L. plantarum* growth on the cantilever, the surface was observed by means of scanning electron microscopy (Nova 200 Nanolab, DualBeam-SEM/FIB, FEI Company).

2.4 Analysis of frequency-amplitude curves

Frequency-amplitude curves were analyzed under the assumption that the cantilevers are damped harmonic oscillators excited at frequency (f) with a corresponding amplitude (A). Frequency-amplitude curves were fitted by a Lorentzian function by means of Eq. 2. The resonance curves of the cantilevers with higher Q -factors are associated with narrower curves. It is thus desirable to have a dynamic AFM resonant system that can achieve an intrinsically high values of the Q -factor (Minary-Jolandan *et al.*, 2012; Ricciardi *et al.*, 2010). Thus, the Lorentzian fit of the resonance curves yielded the f_0 and Q parameters.

For biodetection experiments, the resonance frequency shift (Δf) was obtained as the difference between f_0 (the initial resonance frequency) and f_t (the resonant frequency at time t). The frequency shift as a function of time was fitted by using the Gompertz model which is frequently used to describe active bacterial growth (Buchanan *et al.*, 1997).

All fitting was performed by non-linear least squares regression analysis using Marguardt-Levenberg algorithm. The non-linear regression analysis was done with SigmaPlot software version 11.0 (SYSTAT Inc.USA). The goodness of fit was evaluated by means of the coefficient of determination (R^2).

3 Results and discussion

3.1 Functionalization verified by XPS and CLSM

Figure 1C shows the XPS spectrum of the cantilevers without cleaning or silylanization. A peak is observed at 544 eV that corresponds to oxygen; secondary peaks of Si (112 eV) and C (300 eV) are observed. Finally the spectrum reveals a weak signal C. Figure 1D shows the spectrum of the cantilever after cleaning with piranha solution where the silicon signal decreases because other elements are covering the analyzed surface. The intensities of the C and O peaks increases due to the H_2SO_4 and H_2O_2 contained in the cleaning solution. This is evidence of correct functionalization and silylanization. In XPS, the relative intensities of the photoelectron peaks from a layer are a function of the thickness of the layer of any layers that may cover it (Brundle *et al.* 2010). Figure 1E shows the spectrum of the cantilever after cleaning and silylanization, where it is observed that the intensity of the Si peak has decreased significantly (< 4000 counts/s) and was impacted because XPS can detect elements only from 1 to 8 atomic layers and the cantilever may be coated on its first atomic layers with the silylated compounds that were used (Baer and Engelheard 2010; Gruker *et al.* 1983). Thus the XPS spectrum confirmed the adequate silylanization of the cantilevers.

Additionally, the functionalization with an agarose layer was confirmed by confocal laser scanning microscopy (CLSM). Figure 2A shows a CLSM image of the cantilever without functionalization and Figure 2B shows the cantilever functionalized with agarose film. The fluorescence spectrum showed two emission peaks at 440 nm and 490 nm (data not shown). These peaks may correspond to the fluorescence of silicon and agarose respectively (Chuqiao *et al.* 2011; Zhe *et al.* 2013). In the CLSM images (Figure 2A and 2B), silicon is indicated in red and agarose in green. This confirmed that the cantilevers used as mechanical biosensors with correctly functionalized with agarose.

3.2 Biodetection experiments and biosensor sensitivity

Figure 3A shows the selected resonance frequency spectra for the reference and the incubated cantilevers during the growth kinetics of *L. plantarum*. The resonance frequencies of the control experiments did not showed significant variations during the 4 hours of testing; only small variations of ~ 1 kHz were seen.

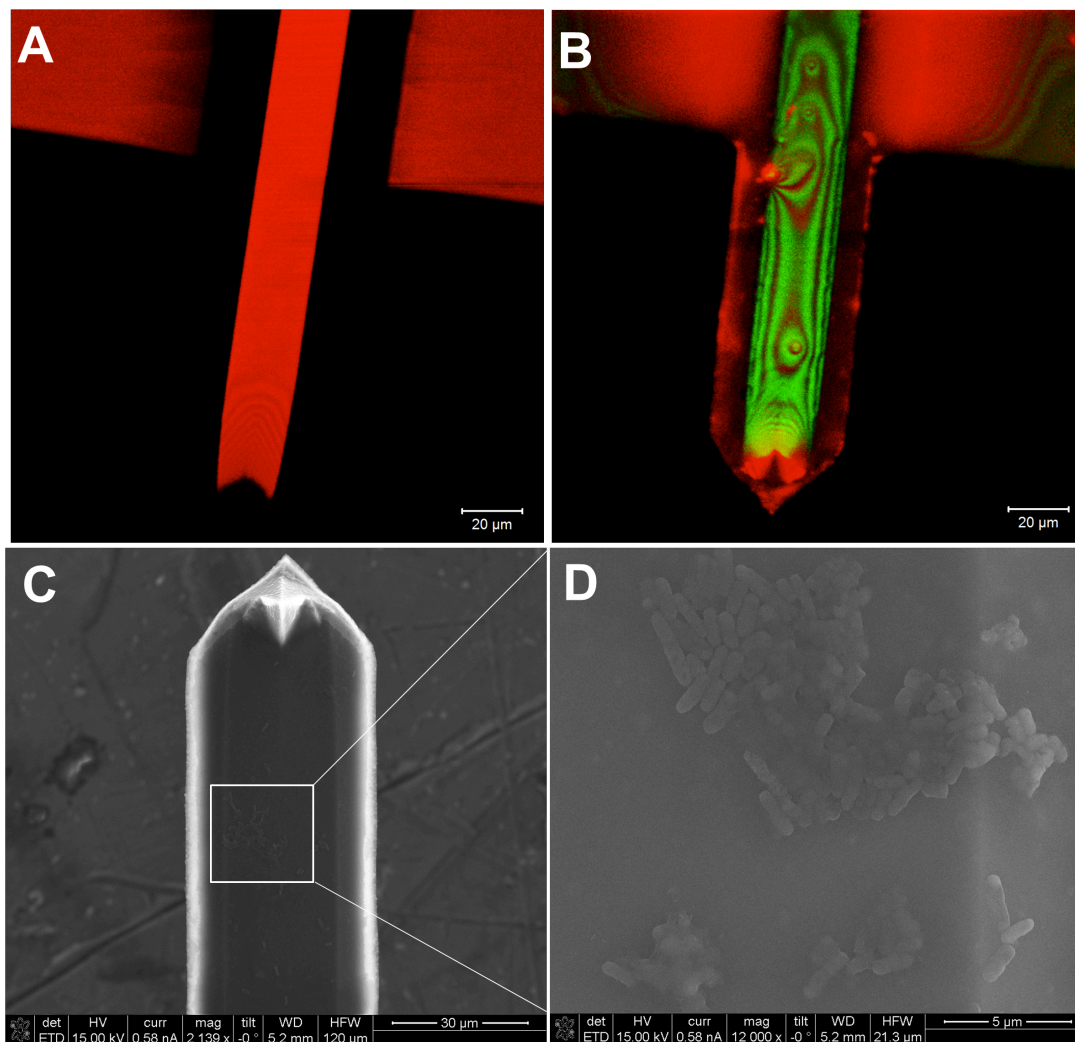


Fig. 2. Confocal laser scanning microscopy images: (A) cantilever without functionalization, and (B) cantilever functionalized by means of silylation and agarose film. Scanning electron microscopy images: (C) cantilever after *L. plantarum* growth kinetic (240 min) and (D) magnification of the cantilever surface.

This can be attributed to the small damping effect by humidified air and from external noise. A continuous decrease of the resonance frequency ($f_0=351.6$ kHz, $f_{120}=349.7$ kHz and $f_{240}=346.8$ kHz) can be observed in the experiments with *L. plantarum* present. This negative shift (around 4.8 kHz) is due to the absorption and active growth of the *L. plantarum* cells on the agarose layer of the cantilever surface. Also, the amplitude (A_{max}) showed a small decrease due to a damping effect caused by cells growth (Nugaeva *et al.* 2005). Figure 2C shows the micrograph of a cantilever after 240 min of *L. plantarum* growth. One can clearly observe that bacterial cells were attached to the biosensor cantilever, as well starting

to form small colonies. *L. plantarum* cells can be seen at a concentration of 10^3 CFU/mL. Dispersed cells are observed along the cantilever, showing the chain bacillary cells (Figure 2D) which is the typical morphology of *L. plantarum*.

During biodetection kinetics, no significant changes were observed in Q -factor values. On average, for all biosensor cantilevers, Q -values were ~ 219 , while the average Q -values obtained for the reference cantilevers was ~ 205 . These results indicate an adequate sensitivity of the cantilevers used in the present work and are in accordance with the data reported by Minary-Jolandan *et al.* (2012) who found Q -values around 450 for cantilevers in air and

between 135 and 90 in water. A high Q -factors is associated with a narrow resonance peak of the AFM response and a good sensitivity (Lübbe *et al.*, 2010; Ricciardi *et al.*, 2010). The goodness of fit of the frequency-amplitude curves using a Lorentzian was high in all experiments ($R^2 > 0.96$) (Figure 3A). Figure 3B shows the behavior of resonance frequency during the tests, obtained by incubating a sensor and a reference cantilever. For these experiments, both cantilevers were coated by an agarose layer, but only the biosensor cantilever was exposed with *L. plantarum*. No decrease in the resonance frequency of the reference cantilever was observed. In contrast, the resonance frequency of the cantilevers with *L. plantarum* decreased significantly during the 4 hours. The mass loading due to the active bacterial growth on the cantilever surface caused the negative shift in its resonance frequency (Nugaeva *et al.*, 2005).

In Figure 3C, the average values of resonance frequency shifts of multiple measurements are plotted as a function of time. For biodetection experiments, the resonance frequency shift decreased on average 5.2 ± 0.8 kHz due to active growth of bacteria during the experiments. Meanwhile, the control experiments saw a resonance frequency shift of, on average, 0.23 ± 0.11 kHz; probably caused by intrinsic variations to external noise, temperature and humidity of the environment. These variations have been reported as a “stabilization period” of the equipment (Nugaeva *et al.*, 2005). The obtained data were fitted using the modified Gompertz model which generates an asymmetric sigmoid curve and describes microorganisms growth in 3 stages, generally categorized as the lag, exponential, and stationary phases (Buchanan *et al.*, 1997; Huang *et al.*, 2008) following Equation (3):

$$\Delta f = ke^{-bt} \quad (3)$$

Here k is the size that can reach the population (kHz), b is the growth rate (min^{-1}) and t is the time. Under any conditions suitable for a microorganism to grow, microbial growth usually exhibits a lag phase, also known as “adaption period”, that denotes when a bacterial culture is introduced into a new environment. This also allows for the bacteria adaptation in the growth medium. The exponential phase follows, during which bacterial cells actively divide and multiply and a logarithmic increase in bacterial population occurs. At the stationary phase, the population of bacteria reaches the maximum density and the growth is partly inhibited due to insufficient nutrition. In the curve plotted in (Figure

3C), a relatively short lag phase can be detected (0-30 min) due to the fact that the microorganisms were in already in a high concentration of 1×10^3 CFU/mL before the incubation time on the cantilevers. An exponential phase could be observed from 30-240 min of biodetection. The stationary phase (which is not observed on Figure 3C) could be observed after 240 min of biodetection if the kinetic is performed at least by 6 h, however 4 h are enough to detect the bacterial growth on the cantilever. Gfeller *et al.* (2005) and Nugaeva *et al.* (2005) show a similar behavior for pathogenic bacteria and fungi.

With regards to sensitivity and according to Equation (1), measuring using cantilevers in dynamic mode cause shifts in the resonant frequency. The primary mechanism of the resonant frequency change occurs through binding induced mass change and/or spring constant change. The effects on the resonant frequency can be expressed most simply by Chen *et al.* (1995):

$$\Delta f = \frac{1}{2} f_0 \left(\frac{\Delta k}{k} - \frac{\Delta m}{m} \right) \quad (4)$$

Here, Δk is the change on the spring constant of cantilever. Equation (4) indicates that the resonant frequency decreases as the cantilever mass increases, but increases if its spring constant increases. Also, the mass or stiffness changes are assumed to occur uniformly over the entire cantilever, a condition which is often not satisfied in practical biosensing applications due to localized binding areas selected for optimum sensor response (Johnson and Mutharasan, 2012). Although various reports have observed resonant frequency changes attributed to adsorption induced changes in the cantilever spring constant or surface stress, even when mass was also attached, the majority of reports to date found the resonant frequency to decrease upon analyte binding and were attributed to an increase in the cantilever mass (Johnson and Mutharasan, 2012; Nugaeva *et al.*, 2005; Sungkanak *et al.*, 2010). Therefore, the stiffness term ($\Delta k/k$) in Equation (4) is often neglected which gives rise to a relation dependent only on mass-change effects. The resulting relation can be restated to define mass change sensitivity:

$$\sigma_b = \frac{\Delta f}{\Delta m} = -\frac{1}{2} \frac{f_0}{m} \quad (5)$$

The mass-change sensitivity has also been defined as the inverse of Equation (5), however in terms of the mass change versus the resonance frequency shift ($\Delta m/\Delta f$), the sensitivity for micro and nanosensors is in the range $\text{ng} \cdot \text{Hz}^{-1}$ to $\text{fg} \cdot \text{MHz}^{-1}$, depending on the

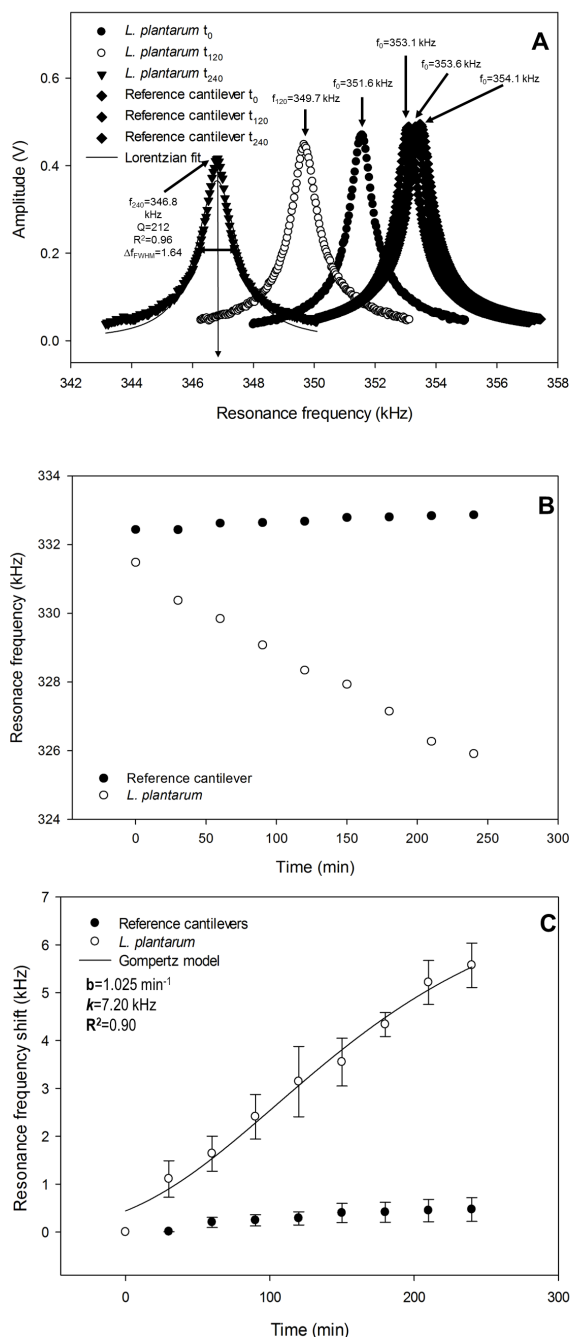


Fig. 3. (A) Resonance frequency spectra reference and biosensor cantilevers, where is show an example fitting with Lorentzian function for yielding f_0 , Q and R^2 parameters. (B) Resonance frequency changes of reference and biosensor cantilevers during growth of *L. plantarum*. (C) Average values and its corresponding standard deviation of resonance frequency shift due to *L. plantarum* active growth of the biosensor and control cantilevers measurements as function of time.

dimensions, sensibility and operating conditions of the sensors (Waggoner and Craighead, 2007). Thus, to evaluate the mass change on a rectangular cantilever, other equations to describe this behavior have been reported (Chen *et al.*, 1995; Sungkanak *et al.*, 2010):

$$\Delta m = \frac{k}{4n\pi^2} \left(\frac{1}{f_1^2} - \frac{1}{f_0^2} \right) \quad (6)$$

Here, n is a correction factor for rectangular cantilevers. Therefore, using Equations (5) and (6), the biosensor sensitivity is calculated to be 383 ± 3 pg/Hz. Taking in account that the mass of a single cell is about 1×10^{-12} g (Zhu *et al.*, 2007), our biosensor can detect ~ 400 cells per 1 Hz signal change. These values agree with Sungkanak *et al.* (2010) who evaluated a microcantilever based cholera sensor with a detection limit of $\sim 1 \times 10^3$ CFU/mL and a mass sensitivity of ~ 146.5 pg/Hz. Also, Zhu *et al.* (2007), who evaluated *Salmonella typhimurium* by using lead titanate zirconate/gold-coated glass cantilevers, obtained a mass sensitivity of 0.5 pg/Hz with concentration sensitivities of 1×10^3 and 500 cells/mL in 2 mL of liquid.

Conclusions

This paper presents the development of microcantilever functionalization for the detection of *L. plantarum* through dynamic force microscopy. Q -factors, f_0 and Δf_0 parameters were suitable to describe and evaluate the signal quality and the changes that occurred to the functionalized cantilevers during the growth of the probiotic microorganisms in air. The Gompertz model was appropriate for describing the growth kinetics of *L. plantarum* in air. Also, SEM observations helped to verify the growth of the probiotic microorganisms on the cantilever surface.

Our results show that cantilevers operated in dynamic mode in humid air allow quantitative and qualitative detection with a sensitivity of 383 ± 3 pg/Hz and a selective detection of ~ 400 cells in 4h. This is faster than the currently applied standard procedures (pour plate technique) for detection of probiotic microorganism. The results obtained are valuable as a proof of concept for the fabrication of portable biosensors based on the mass changes to the cantilevers, for sensing and quality control of probiotic microorganism in the food industry.

Acknowledgments

Angélica Gabriela Mendoza Madrigal would like to thank CONACyT for the scholarship provided. This research was financed through SIP projects at the Instituto Politecnico Nacional and Catedra Coca-Cola para jóvenes investigadores 2011 (Coca Cola-CONACYT).

Nomenclature

A	amplitude (V)
AFM	atomic force microscopy (-)
Amax	maximum amplitude value at f_0 (V)
CFU	colony forming units (-)
CLSM	Confocal laser scanning microscopy (-)
f	resonance frequency (kHz)
f_0	resonance frequency before binding of molecules or growing of microorganisms or initial resonance frequency (kHz)
ft	resonance frequency at present time (kHz)
k	spring constant (N/m)
m	effective mass of cantilever (kg)
MRS	man rogosa sharpe broth (-)
n	correction factor for rectangular cantilevers (-)
Q	quality factor (-)
SEM	Scanning electron microscopy (-)
XPS	X-Ray Photoelectrons Spectroscopy

Greek symbols

Δf	resonance frequency shift (kHz)
Δf_{whm}	full-width at half maximum of the resonance frequency peak (kHz)
Δk	change on the spring constant (N/m)
Δm	added mass to cantilever (kg)
σ_b	sensitivity of biosensor (kg/kHz)

References

- Baer, D. R. and Engelhard, M. H. (2010) XPS analysis of nanostructured materials and biological surfaces. *Journal of Electron Spectroscopy and Related Phenomena* 178-179, 415-432.
- Berquand, A., Xia, N., Castner, D., Clare, B., Abbott, N., Dupres, V., Adriaensen, V. and Dufrene Y. (2005). Antigen Binding Forces of Single Antilysozyme Fv Fragments Explored by Atomic Force Microscopy. *Langmuir* 21, 5517-5523.
- Buchanan, R. L., Whiting, E. C., Damert, W. C. (1997). When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. *Food Microbiology* 14, 313-326.
- Brundle, C. R., Conti G., and Mack, P. (2010). XPS and angle resolved XPS, in the semiconductor industry: Characterization and metrology control of ultra-thin films. *Journal of Electron Spectroscopy and Related Phenomena* 178-179, 433-448.
- Campbell G. A. and Mutharasan, R. (2005). *Escherichia coli* O157:H7 detection limit of millimetersized PZT cantilever sensors is 700 Cells/mL. *Analytical Sciences* 21, 355-357.
- Charalampopoulos, D., Vázquez, J. A. and Pendiella, S. S. (2009). Modelling and validation of *Lactobacillus plantarum* fermentations in cereal-based media with different sugar concentrations and buffering capacities. *Biochemical Engineering Journal* 44, 95-105.
- Chen G. Y., Thundat, T., Wachter, E. A. and Warmack, R. J. (1995). Adsorption-induced surface stress and its effects on resonance frequency of microcantilevers. *Journal of Applied Physics* 77, 3618-3622.
- Chuqiao, T., Xuchu, M. House, A., Kauzlarich M. S. and Louie, A. Y. (2011). PET Imaging and Biodistribution of Silicon Quantum Dots in Mice. *ACS Medicinal Chemistry Letters* 2, 285-288. dx.doi.org/10.1021/ml1002844
- Dhanani, A. S., Gaudana, S. B. and T. Bagchi. (2011). The ability of *Lactobacillus* adhesion EF-Tu to interfere with pathogen adhesion. *European Food Research and Technology* 232, 777-785.
- Gfeller, K. Y., Nugaeva, N., Herner, M. (2005). Micromechanical oscillators as rapid biosensor for the detection of active growth of *Escherichia coli*. *Biosensors and Bioelectronics* 21, 528-533.
- Giessibl, F J (2003). Advances in atomic force microscopy. *Reviews of Modern Physics* 75, 949-983.

- Gunter, R. L., Delinger, W. G., Manyoats, K., Kooser A., and Porter, T. L. (2003). Viral detection using an embedded piezoresistive microcantilever sensor. *Sensors and Actuator 107*, 219-224.
- Gurker, N., Ebel, M. F. and Ebel, H. (1983), Imaging XPS-A new technique, I-principles. *Surface and Interface Analysis 5*, 13-19. doi: 10.1002/sia.740050105
- Huang, L. (2008). Growth Kinetics of *Listeria monocytogenes* in Broth and Beef Frankfurters-Determination of Lag Phase Duration and Exponential Growth Rate under Isothermal Conditions. *JFS E: Food Engineering and Physical Properties 7*, 235-242.
- Ilic, B. Craighead, H.G., Krylov, S., Senaratne, W. Ober, C., Neuzil, P. (2005). Attogram detection using nano electromechanical oscillators. *Journal of Applied Physics 95*, 3694-3703.
- Johnson, B. N., Mutharasan, R. (2012). Biosensing using dynamic-mode cantilever sensors: A review. *Biosensors and Bioelectronics 32*, 1-18.
- Lang, H. P., Hegner, M., Meyer, E., Gerber, C. (2002). Nanomechanics from atomic resolution to molecular recognition based on atomic force microscopy technology. *Nanotechnology 13*, R29-R36.
- Laparra, J. M and Sanz, Y. (2009). Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. *Letters in Applied Microbiology 49*, 695-701.
- Lazcka, O., Del Campo, F. J., Muñoz, F. X. (2007). Pathogen detection: A perspective of traditional methods and biosensors. *Biosensors and Bioelectronics 22*, 1205-1217.
- Lübbe, J., Tröger, L., Torbrügge, S., Bechstein, R., Richter, C. Kühnle, A. and Reichling, M. (2010) Achieving high effective Q -factors in ultra-high vacuum dynamic force microscopy. *Measurement Science and Technology 21*, 1-9.
- Minary-Jolandan, M., Tajik, A., Wang N. and Yu, M-F (2012). Intrinsically high-Q dynamic AFM imaging in liquid with a significantly extended needle tip. *Nanotechnology 23*, 235704.
- Nugaeva, N., Gfeller, K. Y., Backmann, N., Lang, H. P., Düggelin, M., Hegner, M. (2005). Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection. *Biosensors and Bioelectronics 21*, 849-856.
- Raiteri, R., Grattarola, M., Butt, H. J., Sklǎdal, P. (2001). Micromechanical cantilever-based biosensor. *Sensors and Actuators B*, 79, 115-126.
- Ricciardi, C., Canavese, G., Castagna, R., Digregorio, G., Ferrante, V., Marasso, S. Ricci, A., Alessandria V., Rantsiou, K., Cocolin, L. (2010). Online Portable Microcantilever Biosensors for Salmonella enterica Serotype Enteritidis Detection. *Food Bioprocess Technology 3*, 956-960.
- Savran, C. A., Knudsen, S. M., Ellington, A. D. and Manalis, S. R. (2004). Micromechanical detection of proteins using aptamer-based receptor molecules. *Analytical Chemistry 76*, 3194-3198.
- Seo, Y. and Jhe, W. (2008). Atomic force microscopy and spectroscopy. *Reports on Progress in Physics 71*, 016101 (23pp). doi:10.1088/0034-4885/71/1/016101
- Shu, W., Laue E. D., and Seshia, A. A. (2007). Investigation of biotin-streptavidin binding interactions using microcantilever sensors. *Biosensors and Bioelectronics 22*, 2003-2009.
- Su, L., Jia, W., Hou, C., Lei, Y. (2011). Microbial biosensors: A review. *Biosensors and Bioelectronics 26*, 1788-1799.
- Sungkanak, U., Sappat, A., Wisitsoraat, A., Promptmas, C., Tuantranon, A. (2010). Ultrasensitive detection of *Vibrio cholerae* O1 using microcantilever-based biosensor with dynamic force microscopy. *Biosensors and Bioelectronics 26*, 784-789.
- Thévenot, D. R., Toth, K. Durst, R. A., Wilson, G. S. (2001). Electrochemical biosensors: recommended definitions and classification. *Biosensors and Bioelectronics 34*, 636-659.
- Van Dorst, B., Mehta, J., Bekaert, K., Rouah-Martin, E. De Coen, W., Dubrueel, P., Blust, R., Robbens, J. (2010). Recent advances in recognition

- elements of food and environmental biosensors: A review. *Biosensors and Bioelectronics* 26, 1178-1194.
- Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C. (2010). An overview of foodborne pathogen detection: In the perspective of biosensors. *Biotechnology Advances* 28, 232-254.
- Vo-Dinh, T., Cullum, B. M., Stokes, D. L. (2001). Nanosensors and biochips: frontiers in biomolecular diagnostics. *Sensors and Actuators* 74, 2-11.
- Waggoner, P. and Craighead, H. (2007). Micro- and nanomechanical sensors for environmental, chemical, and biological detection. *Lab Chip* 7, 1238-1255.
- Yen, Y. K., Huang, C. Y., Chen, C. H., Hung, C. M., Wu, K. C., Lee, C. K., Chang, J. S., Lin, S. and Huang, L. S. (2009). A novel, electrically protein-manipulated microcantilever biosensor for enhancement of capture antibody immobilization. *Sensors and Actuators B: Chemical* 141, 498-505.
- Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suárez, V., Vinderola, G., Reinheimer, J. and Giraffa, G. (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology* 28, 1033-1040.
- Zhe, J., Jian-Feng, M., Zhi-Heng, Z., Feng X., Run-Cang, S. (2013). Distribution of lignin and cellulose in compression wood tracheids of *Pinus yunnanensis* determined by fluorescence microscopy and confocal Raman microscopy. *Industrial Crops and Products* 47, 212-217.
- Zhu, Q. Shih, W. Y., Shih, W-H. (2007). *in situ*, in-liquid, all-electrical detection of Salmonella typhimurium using lead titanate zirconate/gold-coated glass cantilever at any dipping depth. *Biosensors and Bioelectronics* 22, 3132-3138.