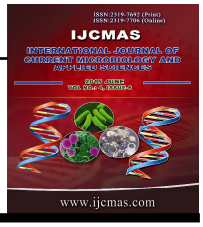
Int.J.Curr.Microbiol.App.Sci (2015) 4(6): 612-620

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 6 (2015) pp. 612-620**
http://www.ijcmas.com



Original Research Article

Influence of pH, temperature and *Ficus odorata* Blanco on the growth of *Lactobacillus salivarius* subspecies *salicinius* JCM 1042 from Filipino breast milk

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Keywords

Probiotics, Prebiotics, Functional foods, Immuno regulation, GIT

A B S T R A C T

The genus *Lactobacilli* is known for its probiotics species that has sparked consumer interest in alternatively addressing their health care needs. In this study, *L. salivarius* subspecies *salicinius* JCM 1042, an identified strain from the breast milk of Filipino women, has a 16S rRNA gene whose partial gene sequencing exhibits a 99% similarity with that of *L. salivarius* CECT 5713, a commonly studied probiotic in the field of gastro-intestinal modulation and immunoregulation. *F. odorata*, a tropical ethnomedicinal plant endemic to the Philippines, is said to be rich in carbohydrates, fibers, and proteins, and may have a possible immunoregulatory effects that, collectively, suggest its prebiotic capabilities. With the objective to observe the growth profile of the *Lactobacilli* in the presence of *F. odorata*, the resulting study reveals that the extract showed no inhibitory against the strain, while the exponential growth of the strain occurs approximately 10 hours earlier in the presence of varying extract concentrations. Grown for 24 hours, the bacterial strain thrives best at pH 5.52 ($\pm$0.02) at 37˚C in a modified MRS broth containing 5mg/ml *F. odorata* crude ethanolic leaf extract. A preliminary co-encapsulation experimentation yielded uncontaminated growth when contents of the lysed beads were plated.

Introduction

Studied for its probiotic usages, *L. salivarius* has been utilized in the development of functional foods, for their beneficial abilities in gastro-intestinal immunoregulation (Albesharat *et al*, 2010; Messaoudi, *et al*., 2013; Million *et al*., 2012; Perez-Caño *et al*., 2010). It has been a well-characterized bacteriocin producer, which directly inhibits the invasion of competing pathogenic strains (Messaoudi, *et al*., 2013). Aside from being a well-characterized bacteriocin producer, Messaoudi *et al*. (2013) elaborates that the species, and congruently, its subspecies are most likely able to manifest similar

biological processes in preventing the onset of diseases from pathogenic bacteria in the gastrointestinal tract. Strains can vary among origins on the human body, and among geographical locations on the planet. To the best knowledge of the researchers, *L. salivarius* subspecies *salicnius* JCM 1042 is the first *L. salivarius* strain to be isolated from the breast milk of Filipino women. Utilizing the BLASTN version 2.2.26+, it's 16S rRNA gene whose partial gene sequencing as accomplished by Qiagen, Korea exhibits significant alignment of 99% similarity, an E-value of 0.0, and genomic score of 2708 bits with that of the complete genome of *L. salivarius* CECT 5713 (ENA, 2002), which indicates its probable novelty.

The *Ficus* genus, consisting of more than 800 species, is a tropical, deciduous, evergreen tree with pharmacological agents contained within almost every plant part that has been linked to anti-cancer agents, antioxidant capabilities, pro-oxidative abilities, and anti-inflammatory properties (Shi *et al*., 2011; Sirisha *et al*., 2010). Although lacking in research, the endemic *Ficus odorata* Blanco, found in the northern provinces of Luzon (Alejandro, 1999), is known to treat allergies, asthmas, tumors, cancers, diarrhea, and diabetes (Tsai *et al*., 2012).

It's unique ß-sitosteryl-3ß- glucopyranoside-6'-*O*-palmitate elucidated by the Chinese researchers, Tsai *et al.* may be connected with probable immuregulatory properties, for ß-sitosterols were able to regulate TNFa, and IL-10 (Alappat, & Awad, 2010). According to Santiago, & Mayor (2014a), a 100g sample of dried *F. odorata* leaves contains 45.7g of total carbohydrates, 36.1g of total dietary fibers, 15.2g of proteins, and trace amounts of Sodium, Potassium, Calcium, and Zinc.

This study aimed to generate a growth profile of *L. salivarius* subspecies *salicinius* JCM 1042 in varying pH, temperature, and *F.odorata* Blanco crude ethanolic leaf extract concentrations with a co-encapsulation experiment in order to preliminarily support its potential to be developed into a probiotic nutraceutical.

Materials and Methods

Samples: Lactobacillus salivarius subspecies salicinius JCM 1042 & Ficus odorata Blanco (Merr.)

The samples originating from the breast milk of lactating Filipino women were obtained from the culture collection of Mr. Reuben Jerome D. Atayde of RCNAS. Stored in cryovials at -80˚C, *L. salivarius* subspecies *salicinius* JCM 1042 was revived, and allowed to grow using Man-Rogosa Sharpe (MRS; Fluka Analytical Sigma-Aldrich Switzerland; Merck, Germany) media in an anaerobic jar (25L Anaero Jar™ Oxoid Hampshire, England) at 37˚C for 24 hours.

F. odorata (Blanco) Merr. leaves were obtained from Barangay San Roman, Buhi, Camarines Sur, and authenticated by the Botany Section of the Philippine National Museum on 6 May 2013. Briefly, one kilogram of leaves was air-dried and ground using a Wiley Mill, percolated with four 24-hour cycles of 10 liters of 95% ethanol, and concentrated using a rotary evaporator (Eyela, USA) at 40˚C before storage (Santiago, & Mayor, 2014b).

Part 1: Morphological & Biochemical characterization of the strain.

Resulting isolated colonies were subjected to the biochemical tests: catalase test, MR/VP test, MRS agar, and TSI agar, and gram-staining in order to verify its genus *Lactobacilli* (Jara *et al*., 2011).

Part 2: Optimization of growth conditions.

The following procedures utilized the microplate reader (Corona Electric Co Ltd: SH-1000 Lab Microplate Reader) for a 96-well microplate, and a modified version of the Resazurin microtiter plate assay based on the study by Khalifa *et al*. (2013). The colorimetric dye, Resazurin, determines cell viability by detecting the reduced form, Resofurin, in the presence of reduction enzymes of the bacterial cell (Elavarasan *et al*., 2013; Khalifa *et al*., 2013). In preparation for pH, temperature, and extract concentration optimization tests, all starter cultures were adjusted to a McFarland Number 1 standard before creating an inoculated MRS broth with a 1:10 dilution. Summarily, in triplicates, 100µl of the inoculated broth was mixed with 100µl of uninoculated broth and read in the microplate reader at 600nm. Immediately after, 30µl of 0.02% resazurin was added, and read once again at the same wavelength.

MRS broths were adjusted to pH 5.52($\pm$0.02), 4.70($\pm$0.02), 7.10($\pm$0.02), and 8.20($\pm$0.02) using 3M HCl and 3M NaOH. The above-mentioned procedure was, then, applied to create the growth profile (Juarez-Tomas *et al*., 2011). Likewise with the same procedure mentioned, MRS broth grown strains at the optimum pH were subjected to the different incubation temperatures, 32˚C, 35˚C, 37˚C (control), and 42˚C using an incubator (MRC Orbital Shaker Incubator) (Juarez-Tomas *et al*., 2011). Lastly, in sterilized 0.5% DMSO, the *F. odorata* ethanolic leaf extract was dissolved to concentrations of 10mg/ml, 6mg/ml, 2mg/ml, and 1mg/ml. The MRS broths were then modified to contain an equal amount of MRS broth and extract in order to achieve final extract concentrations of 5mg/ml, 3mg/ml, 1mg/ml, and 0.5mg/ml within the reaction tube.

For susceptibility testing of *L. salivarius*, in duplicates, an agar overlay disc diffusion test using cephalothin, amikacin, vancomycin, bacitracin, and a disk containing 1mg/ml *F. odorata* extract were applied, and incubated at 37˚C for 24 hours using MRS agar (Gregoret *et al*., 2013; Tulumoglu *et al*., 2013).

For the antimicrobial capabilities of the extract, a set of ATCC strains obtained from USTCMS and grown in Mueller-Hinton Agar (HIMEDIA), was subjected to a disc overlay containing 1mg/ml *F. odorata* extract, and left to incubate at 37˚C for 24 hours.

Part 3: Co-encapsulation of L. salivarius with F. odorata.

A slightly modified methodology originating from a 2014 study was used (Sathyabama *et al*.). Three sets of Calcium-alginate beads were created. The first was simply the formulated Calcium-alginate beads (Set 1), the second contained Calcium-alginate and the *F. odorata* extract (Set 2), and the last contained the Calcium-alginate mix of the bacteria and plant extract (Set 3).

For spectrophotometric purposes at 600 nanometers, precisely one gram of beads, pertaining to any of the three sets was resuspended in nine milliliters of PBS at pH 6.1$\pm$0.2, swirled, and homogenized for 10 minutes at 8000 rpm (IKA® T25 digital ultra Turrax®) (Sathyabama *et al*., 2014). In a 96-well microplate, 100 microliters of the homogenized mixture of beads was added to 100 microliters of distilled water. The corresponding computations on the influence of the extract alone in the bead (Set 2), and the influence of the extract and

the bacteria in the bead (Set 3) with respect to the blank (Set 1) were attained thereafter. Additionally, eight sterilized bottles were filled with precisely one gram of resuspended Set 3 beads, and placed in the incubator at 37˚C. At intervals of three hours, a vial was taken for its contents to be homogenized and spectrophotometrically read. Only the vial labeled 0 hours and 24 hours containing its respective homogenized solutions were diluted as need be for the pour-plate method (Sathyabama *et al*., 2014).

Results and Discussion

Part 1: Morphological & Biochemical Characterization of the Strain.

Upon microscopic observation of the gram staining, as seen in Figure 1, it was clear that the bacteria of interest had the visual physical characteristics of the Lactobacillus specie: small gram-positive, non-branching, coco-bacilli.

Further visual inspection indicated no contamination, for no other bacteria physically manifested itself together with the strain. Only smooth circular pale white colonies ranging in size from half a millimeter to two millimeters persisted on the MRS agar plate.

As noted from the biochemical tests listed in Table 1, the morphological and biochemical characteristics of the strains coincided with the phenotypic characteristics of *Lactobacillus salivarius* listed in Bergey's Manual of Determinative Bacteriology 9th edition (Holt *et al*., 2009), and The Prokaryotes 3rd Edition: A Handbook on the Biology of Bacteria (Hammes, & Hertel, 2006), and shared in the studies conducted by Martin *et al*. (2006), Jara *et al*. (2011), and Tulumoglu *et al.* (2013).

Part 2: Optimization of growth conditions.

Majority of *F. odorata* consists of carbohydrates, and fibers the *Lactobacilli* can use as food in order to proliferate, while its protein content can be used to up-regulate the expression of its genes (Santiago, & Mayor, 2014a). Depicted in Figures 2 to 4, after hourly readings for 24 hours, the bacteria grew best at pH 5.52 ($\pm$0.02), 37˚C, and with an extract concentration of 5mg/ml. Resazurin tests generated red-colored wells, indicating the viability of the cells throughout the assays. Data collected in Figure 4. were subjected to the statistical analyses of one-way ANOVA, Turkey HSD, LSD, and Duncan post hoc tests using SPSS 17.0, and yielded results of significant differences from that of 0mg/ml extract with a $P<0.05$ value.

The plant extract showed no activity, supported by the lack of a zone of inhibition, against *L. salivarius* and selected ATCC bacterial strains in all triplicates as similarly observed in the study of Santiago, & Mayor (2014a). Briefly, both their study, and this current study revealed that amikacin had the strongest activity against all the ATCC bacterial strains, except the *L. salivarius*, Vancomycin had an activity against all except *P. aeruginosa*, and *L. salivarius*, Bacitracin only had an effect on *L. salivarius*, and Cephalothin, and *F. odorata* had none against any of the strains. Probably due to the richness of the nutrients in the plant extract, the concentration of antimicrobial compounds against these pathogenic strains could either be absent or negligible. Having said so, the extract could be beneficial for the growth of not only probiotic bacteria, but also pathogenic bacteria, posing an eventual insidious drawback.

Table.1 Morphological & biochemical characterization of *L. salivarius* subspecies salicinius JCM 1042

Biochemical Tests performed	
Microscopic observation	
Gram staining	Gram positive
Shape	Rod-shaped
Classification	Baccili
Media tests	
MRS media	+
Anaerobic conditions	+
Methyl Red	+
Vogues Proskauer	-
Glucose fermenter	+
Lactose fermenter	+
Sucrose fermenter	+
Catalase test	-
H_2S production	-

Figure.1 Gram stain of L. salivarius sub species salicinius JCM 1042 at 400x magnification

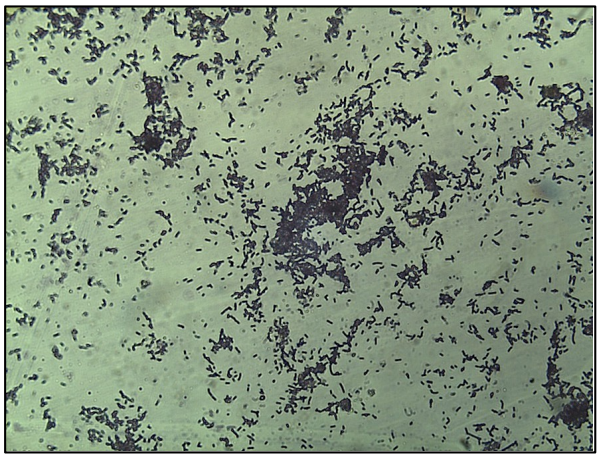


Figure.2 Growth profile of *L. salivarius* using MRS broth at various pH levels at a constant temperature of 37˚C

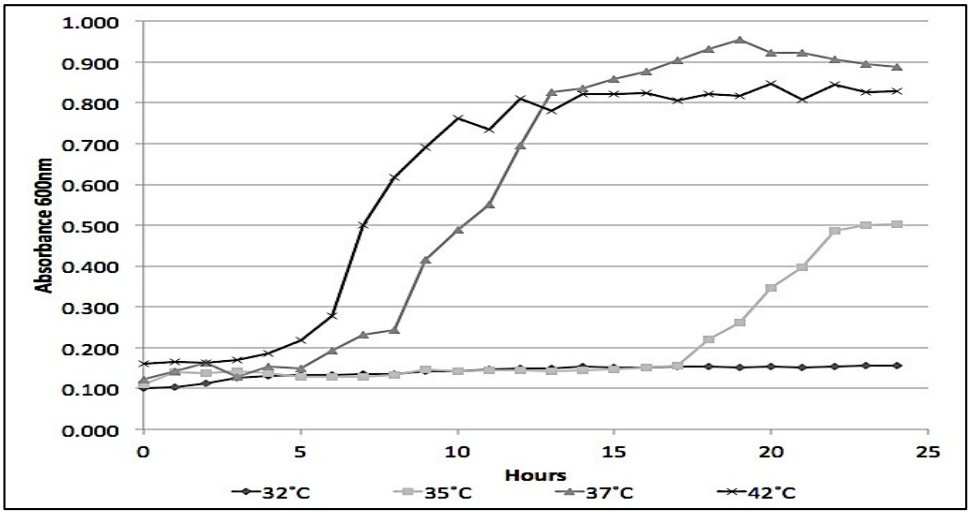


Figure.3 Growth profile of *L. salivairus* using MRS broth at various temperature level at a constant pH level of 5.52 + 0.02

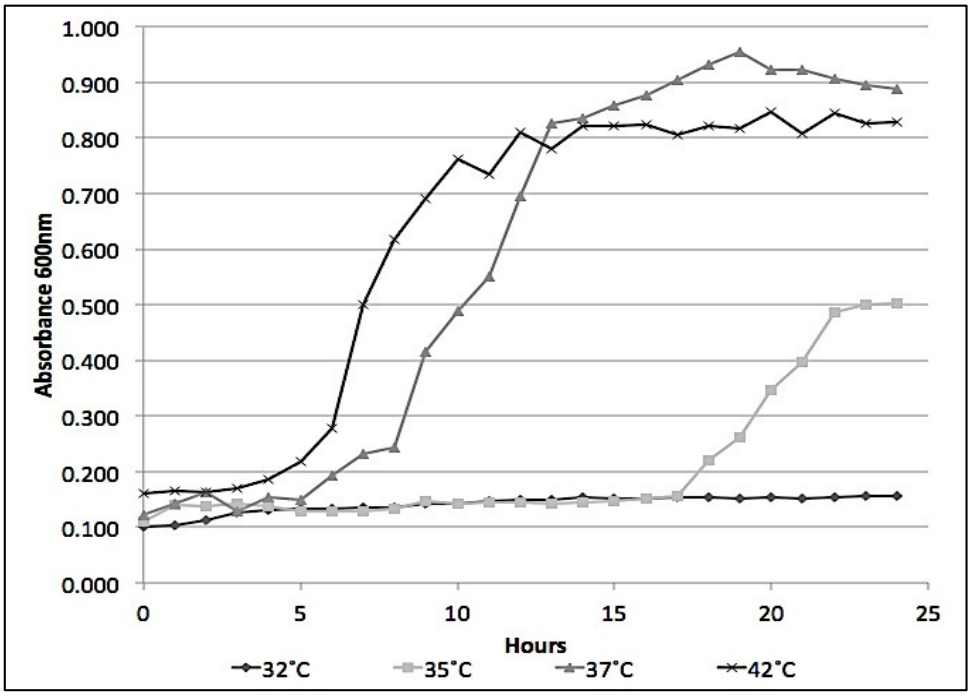
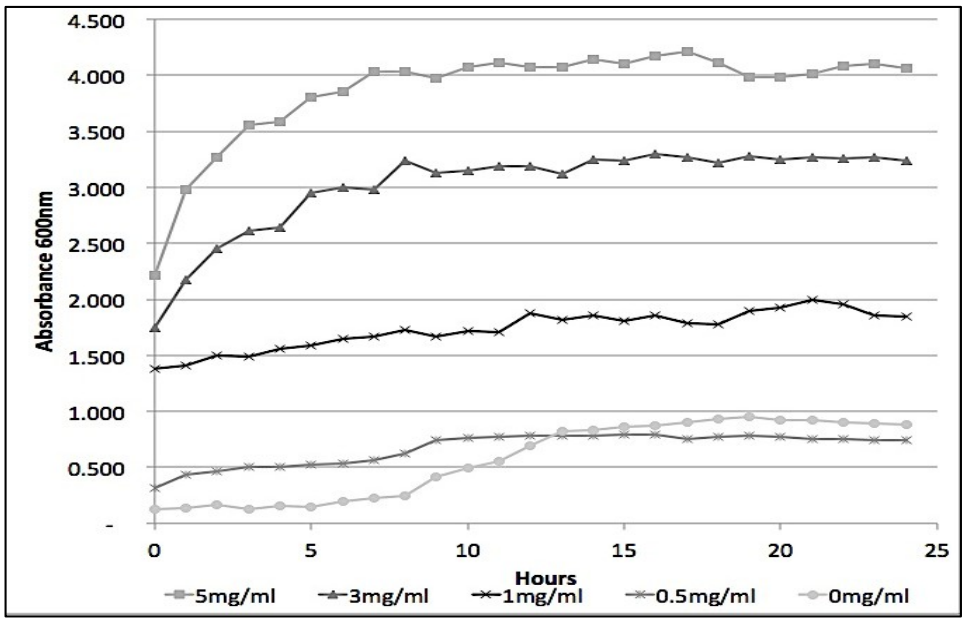


Figure.4 Growth profile of *L. salivarius* using MRS broth at various F. odorata extract concentrations at a pH of 5.52 + 0.02 and 37˚C (5mg/ml, 3mg/ml, 1mg/ml: P<0.05)



Part 3: Co-encapsulation of L. salivarius with F. odorata.

Co-encapsulation of both the bacteria and the plant extract yielded dark green beads of 2.050mm ± 0.025 that grew in size by 1.025mm ± 0.025after one day of incubation at 37˚C, which indicated bacterial growth within the bead. Because the encapsulation method was done aseptically, no contamination was observed during this part of the study. Spectrophotometric readings revealed the gradual increase of bacteria at three-hour intervals for 24 hours. Fortifying this trend were the results of the pour-plate method. Both having a bacterial dilution of 1/100, the plates show that at 0 hours, 107 single colonies formed, while at 24 hours, 282 colonies formed. No contamination was observed during this experimentation

The human breast milk strain under study, *Lactobacillus salivarius* sub-species *salicinius* JCM 1042 shares a 99% similarity with the well-studied probiotic *L. salivarius* CECT 5713 based on its 16s ribosomal RNA partial gene sequencing. Introduction of the *Ficus odorata* extract, which is rich in carbohydrates, fibers, and ions essential to the growth of probiotic bacteria, reveals no inhibitory activity against the strain, but rather a proliferative effect best observed at pH 5.52 (±0.02) and 37 □C with a modified MRS broth containing 5mg/ml of the extract. This leads to the conclusion that *F. odorata* can serve as an effective prebiotic in the production of a probiotic nutraceutical.

Acknowledgements

The authors would like to acknowledge the Faculty of Pharmacy, Department of Biochemistry, and the UST-RNCAS. They, too, would like to extend their gratitude to Assoc. Prof. Ninia I. Calaca for statistical assistance and assurance, and to Mr. Reuben Jerome D. Atayde for providing both the live microorganism used in this study, and the accession number of its sequenced 16s ribosomal RNA profile.

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