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TOPIC

Probiotic Bacteria from Pollen: Selection, Characterization and *in vitro* Inhibition of *Helicobacter pylori*

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JURY

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مـلــخـــص

بكتيريا بروبيوتيك من حبوب الطلع: الانتقاء و التشخيص والتثبيط المخبري للبكتيريا *Helicobacter pylori*

في هذا البحث، تم التحقق من الجودة الميكروبيولوجية لعينات من طلع النحل. تراوح تعداد الفلورا الكلية الهوائية بين 3 و 5 لغ وم م / غ، أما الفطريات فقدرت بين 2.3 و حوالي 7 لغ وم م /غ. كما لوحظت كثافة عالية للمكورات العنقودية الذهبية (وصلت إلى 8.32 لغ وم م / غ). في حين تأرجح تعداد بكتيريا القولون من 3 إلى أكثر من 8 لغ وم م / غ. بالإضافة إلى ذلك، تم الكشف عن الأمعائيات الممرضة و عزلت الفطريات المنتجة للسموم. انتشلت 567 عزلة من بكتيريا حمض اللبن و اعتمادا علي النشاطية العدائية تم تحييد 54 بكتريا حمض اللبن من بين 216 عزلة ذات نشاطية حيوية. حددت سبعة مجموعات مع عضوين آخرين على مستوى التشابه 79 ٪ علي أساس الصفات المظهرية. كما بين التسلسل الجزيئي لمورثات الرنا الريبوزي 16S، أن عشر سلالات من البكتيريا اللبنية تنتمي إلى سبعة أنواع : *Lactobacillus plantarum, Lactobacillus fermentum, Lactococcus lactis, Lactobacillus ingluviei, Pediococcus pentosaceus, Lactobacillus acidipiscis , Weissella cibaria* . شخصت بعد ذلك عشر سلالات من العصيات اللبنية (*Lactobacillus*) علي أساس الميزات المظهرية و الوراثية و التي اختيرت لمزيد من تقييم خصائص التعزيز الحيوي. لم يتمكن من انتشال إلا سلالتين فقط (*Lactobacillus* sp. BH1398 وBH1480) بعد ثلاث ساعات من التجربة في درجة الحموضة 2. ثبت أن الأنواع *Lb. plantarum, Lb. fermentum, Lb.acidophyllus, Lb. pentosus* هي الأكثر مقاومة للظروف القاسية. يبدو أن، 0.3٪ و 0.5٪ من الأملاح الصفراوية لا تؤثر بشكل كبير على بقاء معظم السلالات ، باستثناء *Lactobacillus* sp.BH1398. أبدت كل السلالات مقاومة للكوليستين، الكليندامايسين، الكلورامفينيكول، وسيبروفلوكساسين، لكن معظم السلالات حساسة للمضادات الحيوية، أوكساسيلين، أوكسيتيتراسيكلين، وأموكسيسيلين. تم التحقق في الأخير من أمكانية تأثير *Lactobacillus fermentum* BH1509 الكابح ضد سلالتين من سلالات *Helicobacter pylori*. قيس الفعل القاتل للمحلول الطافي لمستنبت البكتيريا اللبنية. و تم التأكد من إنتاج السلالة BH1509 لمبيد ميكروبي (بكتيريوسين) منخفض الوزن الجزيئي، سمي BAC-F15. بعد تنقية جزئية بالترسيب بسلفات الأمونيوم و كروماتوغرافيا Sephadex G-50، قدرالوزن الجزيئي التقريبي لـ BAC-F15 بعد الهجرة الكهربائية بين 4100 و 6500 دالتن. يبدي هذا البكتيريوسين نشاطية تثبيطية واسعة ضد بكتيريا فساد الأغذية و الممرضة، بما في ذلك البكتيريا إيجابية الجرام مثل *Micrococcus luteus, Staphylococcus aureus, Listeria innocua* وسالبة الجرام، *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Helicobacter pylori* ، *Campylobacter jejuni* . لم يتأثر نشاط السائل الطافي المضاد للبكتيريا بالكاتالاز أو الليباز، لكن ألغي من قبل الإنزيمات المحللة للبروتين مثل بروتيناز K، التربسين و بروناز E. BAC-F15 هو بروتين مقاوم للحرارة. و أظهر نشاطا مثبطا على نطاق واسع من درجة الحموضة (4.0-8.0). و أبدى فعلا قاتلا للميكروبات و اتضح دلك جليا من نمط تأثيره على سلالات *Helicobacter pylori*. إن المبيد الميكروبي والبكتيريا المنتجة له، *Lactobacillus fermentum* BHB1509، قد تكون مضادات ميكروبية مفيدة للسيطرة على الالتهابات المعوية التي تحدثها *Helicobacter pylori*.

كلمات مفتاحية: طلع النحل، مراقبة الجودة النوعية، الأغذية الوظيفية، الملوية البوابية، المعززات الحيوية، الالتهابات المعوية و المعدية، البكتيريوسين، العصيات اللبنية، البكتيريا اللبنية.

ABSTRACT

Probiotic Bacteria from pollen: Selection, Characterization and *in vitro* Inhibition of *Helicobacter pylori*

In this research, microbiological quality control of pollen samples was investigated. TAMC ranged from 3.00 to 5.80 Log CFU/g. TMYC was between 2.3 and about 7 Log CFU/g. However, *Staphylococcus aureus* was recovered with high density (up to 8.32 Log CFU/g). *Enterobactericeae* count ranges from 3.00 to more than 8 Log CFU/g. In addition, samples contain pathogenic members of *Enterobacteriaceae* and potent toxinogenic molds Furthermore, 567 isolates of lactic acid bacteria were recovered from raw bee pollen grains. Based on antagonistic activity, neutralized supernatants of 54 lactic acid bacteria (LAB) cultures from 216 active isolates inhibited the growth of indicator bacteria. They were phenotypically characterized and seven clusters with other two members were defined at the 79% similarity level. Partial sequencing of the 16S rRNA gene of representative isolates, 10 strains were assigned to seven species: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis, Lactobacillus ingluviei*, *Pediococcus pentosaceus*, *Lactobacillus acidipiscis* and *Weissella cibaria*. After that, 10 *Lactobacillus* isolates identified, by phenotypic and genotypic methods, were selected for further evaluation of their probiotic properties. At pH 2, only tow strains (*Lactobacillus* sp. BH1398 and BH1480) that could not recovered after three hours. It seems that, 0.3% and 0.5% of bile salts does not affect greatly the survival of most strains, excluding *Lactobacillus* sp. BH1398. In contrast, in the presence of 1.0% bile salts, survival of five strains was decreased by more than 50 %. In addition, all strains were resistant to colistine, clindamycine, chloramphenicol, and ciprofloxacine, but most of the strains were susceptible to peniciline, oxacillin, Oxytetracyclin, and amoxicillin. Finally, *Lactobacillus fermentum* BH1509 was investigated for its inhibitory effect against two *Helicobacter pylori* strains. The bactericidal activity of spent culture supernatants (SCS) was measured. The strain BH1509 produced a small bacteriocin, designated Bac-F15. After partial purification by ammonium sulfate precipitation and Sephadex G-50 chromatography, the approximate molecular weight of Bac-F15 was estimated by SDS-PAGE between 4100 and 6500 Da. It displayed a wide inhibitory spectrum against food-spoiling bacteria and food-borne pathogens. The antibacterial activity of cell-free culture supernatant fluid was not affected by catalase or lipase but was abolished by the proteolytic enzymes proteinase K, trypsin and pronase E. Bac-F15 was heat stable (10 min at 100°C) and showed inhibitory activity over a wide pH range (from 4.0 to 8.0). It has a bactericidal action as evidenced by its action upon *Helicobacter pylori* strains. The bacteriocin and its producer, *Lactobacillus fermentum* BHB1509, may be useful antimicrobial materials to control gastrointestinal infections caused by *Helicobacter pylori*.

Keywords: Pollen, quality control, functional foods, *Helicobacter pylori*, probiotics, gastrointestinal ulceration, bacteriocin, *Lactobacillus*, lactic acid bacteria.

RESUME

Bactéries Probiotiques de Pollen: Sélection, Caractérisation et L'Inhibition *in vitro* de L'*Helicobacter pylori*

Dans cette recherche, la qualité microbiologique d'échantillons de pollen a été étudiée. La MTAM variait de 3.00 à 5.80 Log UFC/g. Le NTML était entre 2.3 et environ 7 Log UFC/g. Cependant, *Staphylococcus aureus* a été récupéré à haute densité (jusqu'à 8.32 log UFC/g). Le nombre d'entérobactéries varie de 3.00 à plus de 8 Log UFC / g. En outre, les échantillons analysés contiennent des membres pathogènes d'entérobactéries et les moisissures potentiellement toxinogènes, Ensuite, 567 isolats de bactéries lactiques ont été récupérés. Les Surnageants neutralisés des cultures de 54 bactéries lactiques (BAL) parmi 216 isolats actifs inhibent la croissance des bactéries indicatrices. Sur la base de la classification numérique, sept groupes avec deux autres membres ont été définis au niveau de la similitude de 79%. Le séquençage partiel du gène 16S ARNr des isolats représentatifs de chaque groupe, 10 souches ont été attribués à sept espèces: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus ingluvies*, *Pediococcus pentosaceus*, *Lactobacillus piscis* et *Weissella cibaria*. Après cela, 10 isolats de *Lactobacillus* sont identifiés par des méthodes phénotypiques et génotypiques. À pH 2, seulement deux souches (*Lactobacillus* sp. BH1398 et BH1480) qui ne pouvaient pas récupérer au bout de trois heures. *Lb. plantarum*, *Lb. fermentum*, *Lb. acidophilus*, et *L. pentosus* sont révélés être les souches les plus résistantes. Il semble que, 0.3% et 0.5% de sels biliaires n'affecte pas considérablement la survie de la plupart des souches, à l'exclusion de *Lactobacillus* sp. BH 1398. En outre, toutes les souches étaient résistantes à la colistine, la clindamycine, le chloramphénicol et la ciprofloxacine, mais la plupart des souches étaient sensibles à la peniciline, oxacilline, l'oxytétracycline, et l'amoxicilline. Enfin, *Lactobacillus fermentum* BH1509 a été étudié pour son effet inhibiteur contre deux souches d'*Helicobacter pylori*. La souche BH1509 produit un petit bactériocine, désigné Bac-F15. Après purification partielle par précipitation au sulfate d'ammonium et Chromatographie sur Sephadex G-50, le poids moléculaire approximatif de Bac-F15 a été estimé par SDS-PAGE, entre 4100 et 6500 Da. Il présente un large spectre d'action. L'activité antibactérienne du surnageant de la culture bactérienne n'a pas été affectée par la catalase ou de la lipase, mais a été supprimée par les enzymes protéolytiques tel que la protéinase K, la trypsine et la pronase E. Bac-F15 était stable à la chaleur (10 min à 100°C) et il a montré une activité inhibitrice dans un large intervalle de pH (4.0 à 8.0). Il a une action bactéricide comme en témoigne son action sur les souches *Helicobacter pylori*. La bactériocine et son producteur, *Lactobacillus fermentum* BHB1509, peuvent être des agents antimicrobiens utiles pour lutter contre les infections gastro-intestinales causées par *Helicobacter pylori*.

Mots clès: Pollen, contrôle de qualité, aliments fonctionnelles, *Helicobacter pylori*, probiotiques, ulcération gastrointestinal, bacteriocin, *Lactobacillus*, bactéries lactiques

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
GIT	Gastrointestinal Tract
H.	*Helicobacter*
HPLC	High performance Liquid chromatography
Lb.	*Lactobacillus*
LAB	Lactic Acid Bacteria
MRS	De Mamm, Rogosa and Sharpe
PCR	Polymerase chain reaction.
PFGE	Pulsed-field gel electrophoresis
rDNA	Ribosomal Deoxyribonucleic Acid
UPGMA	Unweighted pair group algorithm with arithmetic averages
ARDRA	Amplified ribosomal dna restriction analysis.
TAMC	Total aerobic mesophillic count
CFU	Colony forming units
TMYC	Total mold and yeast count

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II. Experimental Section

GENERAL INTRODUCTION

The use of functional bacterial cultures, a novel generation of microbial cultures that offers functionalities beyond technological properties, is being explored. For instance, lactic acid bacteria (LAB) are capable of inhibiting various microorganisms and display crucial antimicrobial properties with respect to safety and health enhancement. In addition, it has been shown that some strains of LAB possess interesting health-promoting properties; one of the characteristics of these probiotics is the potential to combat gastrointestinal pathogenic bacteria such as *Escherichia coli, Salmonella* and *Helicobacter pylori* (Navarro-Rodriguez *et al.*, 2013).

Helicobacter pylori infection is the major cause of chronic gastritis and peptic ulcer disease and is a risk factor for gastric cancer in humans. This infection is extremely common throughout the world, and its prevalence increases with age and lower socioeconomic status. Combinations of several drugs are now widely used for the eradication of *H. pylori*. Numerous clinical trials have indicated that eradication of *H. pylori* by treatment that includes bismuth or antisecretory drugs combined with antibiotics leads to healing of gastritis and drastically decrease the rate of peptic ulcer relapse. However, a limited number of antibiotics can be used, and drug resistance jeopardizes the success of treatment (O'Connor et al., 2013). Therefore, a search for new antimicrobial agents is warranted. Several authors have previously reported that certain probiotic bacteria, such as *Lactobacillus* spp., exhibit inhibitory activity against *H. pylori in vitro* and *in vivo*. Various studies regarding the antagonistic effect of certain LAB strains, i.e., *Lactobacillus acidophilus*, *Lb. rhamnosus* GG, *Lb. salivarius*, and *Lb. gasseri*, against *Helicobacter pylori* have been reported (Midolo *et al.*, 1995; Coconnier *et al.*, 1998; Canducci *et al.*, 2000; Armuzzi, 2001; Sakamoto *et al.*, 2001; Kamiji and de Oliveira, 2005; O'Connor *et al.*, 2009; Panpetch *et al.*, 2011). The anti-*H. pylori* effect is reported to organic acid production, competitive inhibition for the binding sites of mucous cells, and immunomodulation. However, the synthesis of bacteriocin-like compounds has rarely been associated with these antagonistic effects. In fact, dairy products, meats, fishes, animals and humans and plant materials (fruits and vegetables) are conventional sources for potent probiotic lactic acid bacteria.

Beehive products (honey, pollen, propolis and royal jelly) are natural functional foods that have gained increased attention in

society. Pollen, the male gametophyte of flowering plants, is a high-energy material collected by honeybees and other insects and stored as a food reserve. Pollen has been used traditionally by humans as a supplementary food and in alternative medical treatments. It has been used medically in prostatitis, bleeding stomach ulcers and some infectious diseases (Linskens and Jorde, 1997).

Because of its complex content, bee-pollen has a very important nutritional value in the human diet (Gergen *et al.*, 2006). Since the middle of the last century, the bee-pollen microflora has been investigated (Chevtchik, 1950; Gilliam, 1979 a; 1979 b; Gilliam *et* al. 1989; Gilliam *et al.* 1990; Belhadj *et al.,* 2010; Belhadj *et al.,* 2012). However, little is known about the occurrence of lactic acid bacteria (LAB), and the roles they play, in pollen. Only a few reports are available in the literature considering this field. Occurrence of Lactobacilli in pollen was reviewed by Gilliam (1979a). In another study, Vásquez and Olofsson (2009) identified lactobacilli isolated from pollen grains. These isolates were identified based on PCR and 16S rRNA gene sequencing. Other LAB representative genera (*Lactococcus, Lactobacillus, Pediococcus, Enterococcus* and *Leuconostoc*) were recovered and identified based on phenotypic traits (Belhadj *et al.,* 2010). In the latter investigation, *in vitro* studies indicated that several strains inhibit Gram-positive and Gram-negative pathogenic bacteria. Additionally, several members of LAB are known to produce antibacterial substances such as organic acids and bacteriocins. Antagonism towards undesirable microorganisms is an important criterion for LAB being used as bio-preservations or biocontrol agents. It seems that pollens are a suitable ecological niche for various microorganisms and an important source for the isolation of new strains belonging to the LAB group with antagonistic activity against harmful bacteria. It is well established that probiotic traits are strain dependent aspects. However, when the probiotic microorganism is being to be delivered for the gut, tolerance to stomach and intestinal harsh conditions is needed. Over the past few decades, probiotic LAB have been very intensively investigated. Most of the LAB isolated from raw plants and plant products constitute an important source of new functional bacteria that can play roles in GI of humans and animals or food processing (Leroy and De Vuyst, 2004).

In searching for interesting strains with probiotic potential, studies on natural plant derived products as a source of new isolates are rapidly accumulating.

The present work describes isolation and phenotype-based numerical clustering of LAB isolated from pollen grains collected in some Algerian areas; 16S rRNA gene sequencing-based characterization of selected strains was investigated. Also, their antagonistic activity against Gram-positive and Gram-negative bacteria was evaluated. Furthermore, functional characteristics of potential probiotic *Lactobacillus* strains isolated from bee pollen grains were investigated. The isolates were preliminarily selected on the basis of acid and bile tolerance, and the selected isolates were further screened for various functional properties such as antimicrobial activity against pathogenic bacteria, and hydrophobicity ability. In addition, the probiotic candidate isolates were characterized by means of phenotypic and genotypic (16S rRNA gene sequencing). In addition, the susceptibility of *H. pylori* to *Lb. fermentum* BH1509, a strain isolated from pollen, and to its supernatant as well as the characterization of the antimicrobial protein secreted by this bacterium was also addressed. The final purpose is to find a possible alternative to antibiotic treatments for *H. pylori* strains. The possibility that isolated bacteriocin produced by *Lb. fermentum* could have the potential to inhibit the growth of *H. pylori* as well as some characteristics of the secreted protein is discussed. Our attention has been focused also on the microbial content of pollen (from a quality control point of view) and pathogenic members of *Enterobacteriaceae* as well as fungi were also isolated and charactetized.

I.

Review of the Literature

Chapter 01

Probiotic LAB

1. Probiotics: characteristics, selection criteria and modulation of gut defence barrier

1.1 *An overview on lactic acid bacteria*

Lactic acid bacteria (LAB) are gram-positives, nonsporing, catalase negatives in the absence of porphorinoids, aerotolerant, acid tolerant, organotrophic, and strictly fermentative rods or cocci, producing lactic acid as a major end product. They lack cytochromes and are unable to synthesize porphyrins. Catalase and cytochromes may be formed in the presence of hemes and lactic acid can be further metabolized, resulting in lower lactic acid concentrations. Cell division occurs in one plane, except pediococci. The cells are usually nonmotile. They have a requirement for complex growth factors such as vitamins and amino acids. An unequivocal definition of LAB is not possible (Axelsson 2004). They produce either a mixture of lactic acid, carbon dioxide, acetic acid and/or ethanol (heterofermentatives) or almost entirely lactic acid (homofermentatives) as the major metabolic end product (Kandler et al, 1986; Schillinger et al, 1987). They are mesophilic, some can grow below 5°C and others at temperatures as high as 45°C. Some LAB can grow in acidic pH (3, 2) and others in alkalin pH (9,6) with most growing in the pH range 4,0-9,5 (Jay,2000). The DNA of LAB has a G + C content below 55 mol%, and so are phylogenetically included in the so-called Clostridium branch of Gram positive bacteria (Mavhungu, 2005).

1.2 *Taxonomic status of LAB*

Since the pioneer work of Orla-Jenson (1919), LAB had recognized extensive taxonomic studies. Based on sugar fermentation and growth at specific temperatures, the author classified lactic acid bacteria into seven genera. The sixth edition of Bergy's manual of systematic bacteriology described eight genera: *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus, Carnobacterium, Enterococcus, Lactococcus* and *Vagococcus* (Jay, 1992).

LAB are grouped into the *Clostridium* branch of gram positive bacteria possessing a relationship to the bacilli, while *Bifidobacterium* belongs to the Actinomycetes. They are grouped in one order, six families and thirty two genera (Table 1) (Axelsson, 2004).

Bifidobacteria, which also produce lactate and acetate as the end-products of their carbohydrate metabolism, is often listed along with the lactic acid bacteria sensu stricto, have a unique pathway of hexose fermentation that differs from metabolic routes of LAB in part. In the other part, they have a G+C content over 50 mol % and

belongs to the *Actinomyces* subdivision of the Gram positive bacteria (comprising also *Atopobium*, *Brevibacterium*, *Propionibacterium*, and the microbacteria) and therefore is only quite distantly related to the genuine lactic acid bacteria. (Vandamme *et al*, 1996; Wood and Holzapfel, 1995). The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis of phenotypic features do not correspond with the phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE), are regarded important for specific characterization and detection of LAB strains (Gevers , 2001; Holzapfel , 2001). The phylogenetic tree of the LAB and nonrelated Gram-positive genera *Bifidobacterium* and *Propionibacterium* is presented in figure 1.

1.3 *Occurrence of lactic acid bacteria in nature*

The occurrence and distribution of LAB in nature are related to their fastidious requirements and type of energy generation (purely by fermentation). They are usually associated with nutritionally rich habitats. LAB were first isolated from milk (Metchnikoff, 1910; Sandine , 1972; Carr , 2002). They can be found in fermented products as meat, milk products, vegetables, beverages and bakery products. (Aukrust and Blom, 1992; Harris , 1992; Gobbetti and Corsetti, 1997; Caplice and Fitzgerald 1999; Jay, 2000; Lonvaud-Funel, 2001; O'Sullivan , 2002; Liu, 2003). LAB occurs naturally in soil, water, manure, sewage, silage and plants. (Holzapfel *et al*, 2001). They are part of the microbiota on mucous membranes, such as the intestines, mouth, skin, urinary and genital organs of both humans and animals, and may have a beneficial influence on these ecosystems (Schlegel, 1996). However, certain LAB, especially the streptococcal species (excluding *St. thermophillus*), are pathogenic. In addition to these true pathogens, certain other LAB, for example species in the genera *Lactobacillus*, *Enterococcus*, *Weissella* and *Leuconostoc*, may be involved in opportunistic infections (Devriese , 2000; Björkroth and Holzapfel, 2003).

As fastidious organisms, LAB requires a variety of amino acids, B vitamins, purine and pyrimidine bases for growth. In many environments, they obtain these amino acids through proteolytic activity. This activity is responsible for flavor development during cheese production (Dykes, 1991; Jay, 2000).

Plant foods and vegetables are strongly recommended in the human diet since they are rich in antioxidants, vitamins, dietary fibers and minerals. The major part of the vegetables consumed in the human diet are fresh, minimally processed, pasteurized or cooked by boiling in water or microwaving. Minimally processed and, especially, fresh vegetables have a very short-shelf life since they are

Table 1: Taxonomic outline of lactic acid bacteria of the *Clostridium* branch (Garrity ,2005)

Phylum **Class** **Order**	**Family**	**Genus**
"Firmicutes" **"Bacilli"** **"Lactobacillales"**	**I. Lactobacillaceae**	**I. *Lactobacillus*** **II. *Paralactobacillus*** **III. *Pediococcus***
	II. "Aerococcaceae"	**I. *Aerococcus*** **II. *Abiotrophia*** **III. *Dolosicoccus*** **IV. *Eremococcus*** **V. *Facklamia*** **VI. *Globicatella*** **VII. *Ignavigranum***
	III. "Carnobacteriaceae"	**I. *Carnobacterium*** **II. *Agitococcus*** **III. *Alkalibacterium*** **IV. *Allofustis*** **V. *Alloiococcus*** **VI. *Desemzia*** **VII. *Dolosigranulum*** **VIII. *Granulicatella*** **IX. *Isobaculum*** **X. *Lactosphaera*** **XI. *Marinilactibacillus*** **XII. *Trichococcus***
	IV. "Enterococcaceae"	**I. *Enterococcus*** **II. *Atopobacter*** **III. *Melissococcus*** **IV. *Tetragenococcus*** **V. *Vagococcus***
	V. "Leuconostocaceae"	**I. *Leuconostoc*** **II. *Oenococcus*** **III. *Weissella***
	VI. Streptococcaceae	**I. *Streptococcus*** **II. *Lactococcus***

Principal genera of LAB are underlined

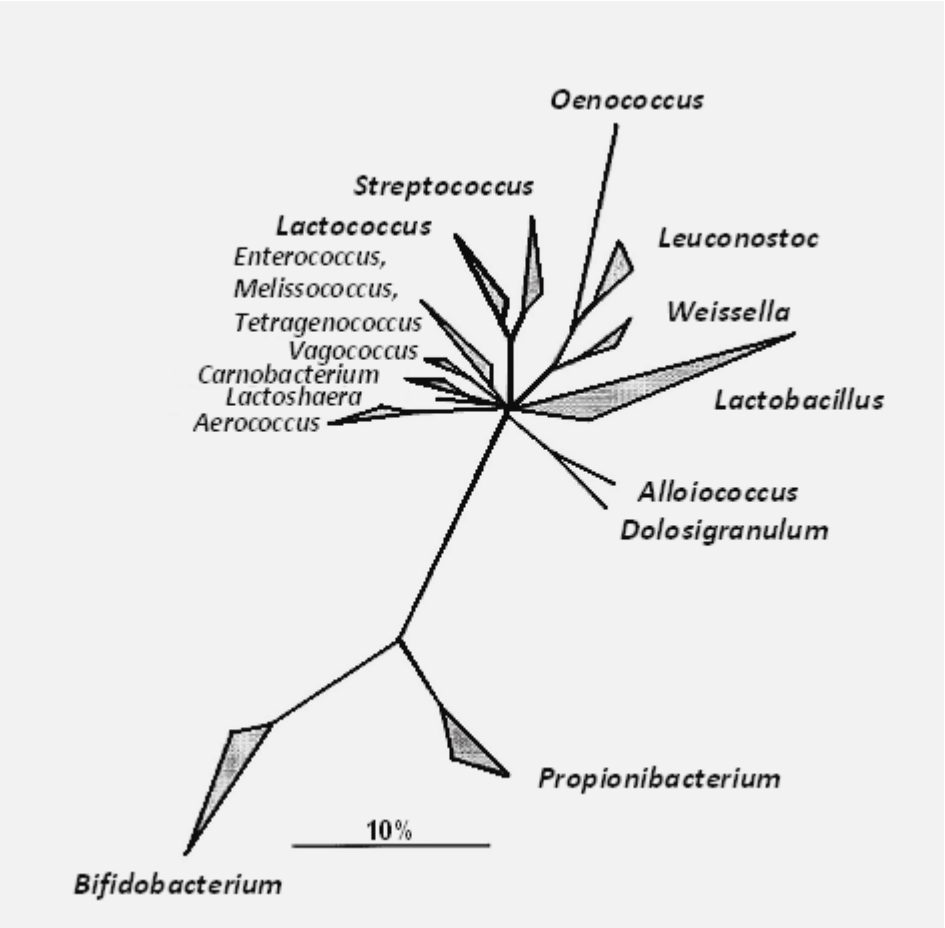


Figure 1: Consensus tree based on comparative analysis of the 16S rRNA gene, showing the major phylogenetic groups of lactic acid bacteria with low mol% of guanine plus cytosine in the DNA and the nonrelated Gram-positive genera *Bifidobacterium* and *Propionibacterium* (Holzapfel , 2001).

subjected to rapid spoilage due to moulds, yeasts and bacterial action. Among other microbial groups, the occurrence of LAB on plants is not new (Langston and Bouma, 1960a; 1960b; Mundt and Hammer, 1968).

As plant product, bee-pollen was used for thousands of years. Historically, it has been referred to as a complete food. It was used as a food and tonic in Chinese, Egyptian, Greek, European, Inca, and North American Indian civilizations. In these ancient cultures bee-pollen was surrounded by mystique; offering long life, improved sexual prowess and prolonged wellness (Crane, 1999). Scientists have studied pollen in modern times and have confirmed that it is a near perfect food. As well as other apicultural products, bee-pollen has gained increased attention for its therapeutic properties; as antimicrobial, anti-caryogenic, immunomodulatory (reviewed in Carpes , 2007) and antioxidant effects (Almaraz-Abarca , 2004; Carpes , 2007). Other applications of pollen include: its use as a functional food (Bogdanov, 2004), prevention of bone loss (Yamaguchi , 2006) and benign prostatic hypertrophy (BPH) treatment (Shoskes, 2002). For these potential applications, bee-pollen was subjected for extensive and various chemical, biochemical and microbiological studies. Also, bee-pollen and bee-bread varies widely in protein content and amino acid composition (Barbier, 1970; Todo and Bretherick, 1940), fatty acids, vitamins and minerals (Loper 1980) and in enzyme constitution. These chemical and biochemical changes have often been postulated to be the result of microbial action, principally a lactic acid fermentation caused by bacteria and yeasts (Reviewed in Gilliam, 1979 a). However, the sources of the microbial flora of bee-bread can be attributed principally to honey-bees, wither, comb cells of the hive, nectar, water and bee-pollen. Since the middle of the last century, bee-pollen microflora was investigated and various fungal, yeast and bacterial species were characterized (Chevichik, 1950; Gilliam, 1979 a; 1979 b; Gilliam 1989; Gilliam 1990). For the best of our knowledge, scarce studies are available in the literature addressing the occurrence of lactic acid bacteria in bee-pollen.

1.4 *Application of LAB as Probiotic agents*

The word ‘‘probiotics’’ was initially used as an anonym of the word ‘‘antibiotic’’. It is derived from Greek words pro and biotos and translated as ‘‘for life’’. The origin of the first use can be traced back to Kollath, who used it to describe the restoration of the health of malnourished patients by different organic and inorganic supplements. Later, Vergin proposed that the microbial imbalance in the body caused by antibiotic treatment could have been restored by a probiotic rich diet; a suggestion cited by many as the first reference to probiotics as they are defined nowadays. Similarly, Kolb recognized detrimental effects of antibiotic therapy and proposed the prevention by probiotics (Vasiljevic and Shah, 2008) Later on, Lilly and Stillwell (1965) defined probiotics as “…microorganisms promoting the growth of other microorganisms”. Following recommendations of a FAO/WHO working group on the evaluation of probiotics in food (2002), *probiotics*, are live microorganisms that,

when administered in adequate amounts, confer a health benefit on the host (Sanders, 2008; Schrezenmeir and De Vrese, 2001). The idea of health-promoting effects of lactobacilli is by no means new, as Metchnikoff as early as 1910 proposed that lactobacilli may fight against intestinal putrefaction and contribute to long life (Metchnikoff 1910). Such microorganisms may not necessarily be constant inhabitants of the gut, but they should have a "…beneficial effect on the general and health status of man and animal" (Holzapfel *et al*, 2001). Other definitions advanced through the years have been restrictive by specification of mechanisms, site of action, delivery format, method, or host. Probiotics have been shown to exert a wide range of effects. The mechanism of action of probiotics (e.g., having an impact on the intestinal microbiota or enhancing immune function) was dropped from the definition to encompass health effects due to novel mechanisms and to allow application of the term before the mechanism is confirmed. Physiologic benefits have been attributed to dead microorganisms. Furthermore, certain mechanisms of action (such as delivery of certain enzymes to the intestine) may not require live cells. However, regardless of functionality, dead microbes are not probiotics. (Sanders, 2008). In relation to food, probiotics are considered as "viable preparations in foods or dietary supplements to improve the health of humans and animals". According to these definitions, an impressive number of microbial species are considered as probiotics. (Holzapfel *et al*, 2001). Commercially, however, the most important strains are lactic acid bacteria (LAB) (Table 2).

1.5 *Criteria for the selection of Probiotics*

Many in vitro tests are performed when screening for potential probiotic strains. The first step in the selection of a probiotic LAB strain is the determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain. All these characteristics have important consequences on the selection of the novel strains (Morelli, 2007). An FAO/WHO (2002) expert panel suggested that the specificity of probiotic action is more important than the source of microorganism. This conclusion was brought forward due to uncertainty of the origin of the human intestinal microflora since the infants are borne with virtually sterile intestine. However, the panel also underlined a need for improvement of *in vitro* tests to predict the performance of probiotics in humans. While many probiotics meet criteria such as acid and bile resistance and survival during gastrointestinal transit, an ideal probiotic strain remains to be identified for any given indication. Furthermore, it seems unlikely that a single probiotic will be equally suited to all indications; selection of strains for disease-specific indications will be required. (Shanahan, 2003).

The initial screening and selection of probiotics includes testing of the following important criteria: phenotype and genotype stability, including plasmid stability; carbohydrate and protein utilization patterns; acid and bile tolerance and survival and growth; intestinal epithelial adhesion properties; production of antimicrobial substances; antibiotic resistance patterns; ability to inhibit known pathogens,

spoilage organisms, or both; and immunogenicity. The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization (Tuomola *et al* 2001). The table below (Table 3) indicates key creteria for sellecting probiotic candidat for commercial application.

Table 2: Microorganisms whose strains are used or considered for use as probiotics (Leroy 2008)

***Lactobacillus* sp.**	***Bifidobacterium* sp.**	**Other Lactic Acid Bacteria**	**Other microorganisms**
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis* [a]	*Bacillus cereus*[a,b]
L. amylovorus	*B. animalis subsp. animalis*	*Enterococcus faecium* [a]	*Bacillus subtilis* [b]
L. brevis	*B. animalis* subsp. *lactis*	*Lactococcus lactis*	*Clostridium butyricum*
L. casei	*B. bifidum*	*Leuconostoc mesenteroides*	*Escherichia coli* [b]
L. crispatus	*B. breve*	*Sporolactobacillus inulinus* [a]	*Propionibacterium Freudenreichii* [a,b]
L. curvatus	*B. longum*	*Streptococcus thermophilus*	*Saccharomyces cerevisiae* [b]
L. delbrueckii subsp.*bulgaricus*			*Saccharomyces Boulardii* [b]
L. fermentum			
L. gallinaruma			
L. gasseri			
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			
L. salivarius			

[a] mainly applied in animals

[b] mainly applied in pharmaceutical preparations

It is of high importance that the probiotic strain can survive the location where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the strain can proliferate and colonise at this specific location. Probably only host-specific microbial strains are able to compete with the indigenous microflora and to colonise the niches. Besides, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. So, the host must be immuno-tolerant to the probiotic. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. It goes without saying that a probiotic has to be harmless to the host: there must be no local or general pathogenic, allergic or mutagenic/carcinogenic reactions provoked by the microorganism itself, its fermentation products or its cell components after decrease of the bacteria (Desai, 2008).

Table 3: Key and desirable criteria for the selection of probiotics in commercial applications (Vasiljevic and Shah, 2008).

Safety criteria	Origin Pathogenicity and infectivity Virulence factors—toxicity, metabolic activity and intrinsic properties, i.e., antibiotic resistance
Technological criteria	Genetically stable strains Desired viability during processing and storage Good sensory properties Phage resistance Large-scale production
Functional criteria	Tolerance to gastric acid and juices Bile tolerance Adhesion to mucosal surface Validated and documented health effects
Desirable physiological criteria	Immunomodulation Antagonistic activity towards gastrointestinal pathogens, i.e., *Helicobacter pylori*, *Candida albicans* Cholesterol metabolism Lactose metabolism Antimutagenic and anticarcinogenic properties

When probiotic strains are selected, attributes important for efficacy and technological function must be assessed and a list of characteristics required for all probiotic functions is required. Basic initial characterization of strain identity and taxonomy should be conducted, followed by evaluation with validated assays both in studies of animal models and in controlled studies in the target host. In vitro assays are frequently conducted that have not been proved to be predictive of in vivo function. Technological robustness must also be determined, such as the strain's ability to be grown to high numbers, concentrated, stabilized, and incorporated into a final product with good sensory properties, if applicable, and to be stable, both physiologically and genetically, through the end of the shelf life of the product and at the active site in the host. Assessment of stability can also be a challenge, since factors such as chain length and injury may challenge the typical assessment of colony-forming units, as well as in vivo function (Sanders, 2008). Dose levels of probiotics should be based on levels found to be efficacious in human studies. One dose level cannot be assumed to be effective for all strains. Furthermore, the impact of product format on probiotic function has yet to be explored in depth. The common quality-control parameter of colony-forming units per gram may not be the only parameter indicative of the efficacy of the final product. Other factors, such as probiotic growth during product manufacture, coating, preservation technology, metabolic state of the probiotic, and the presence of other functional ingredients in the final product, may play a role in the effectiveness of a product. More research is needed to understand how much influence such factors have on in vivo efficacy.

1.5.1 *Acid and Bile Tolerance*

More than two liters of gastric juice with a pH as low as 1.5 is secreted from cells lining the stomach each day, providing a normally-effective, high-acid barrier against entrance of viable bacteria into the GIT. Before reaching the stomach, probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity. However, the effect of gastric pH (pH 1.5-3.0) on bacterial viability and in preventing bacterial colonization of the small intestine is well-studied. Consequently, any probiotic organism that is to survive transit through the stomach must have a high acid tolerance. In typical acid tolerance tests, the viability of candidate probiotic organisms is determined by exposing them to low pH in a buffer solution or medium for a period of time, during which the number of surviving bacteria remaining is determined (Simon and Gorbach, 1987; Heatley and Sobala, 1993; Chou and Weimer, 1999; Çakır 2003).

Several *in vitro* studies (Hood and Zottola, 1988; Charteris, 1998) showed a variation in the resistance of lactic acid bacteria to lower pH (2.0 for 60 min). Eight cultures including *Lactobacillus* and *Pediococcus* strains were exposed to low pH (pH 1.0 to 5.0) conditions of stomach for 1 h. The number of surviving bacteria was decreased from the inoculated level of 7.4-7.6 log cfu/mL to < 4 log cfu/mL at pH 1.0 and pH 2.0, whereas pH 4.0 and 5.0 did not affect the viability (Erkkila and

Petaja, 2000). In other study, Ruiz-Moyano , (2008) showed that only 51 out of 312 pre-selected LAB strains, including *Lactobacillus*, *Pediococcus*, *Enterococcus*, isolated from Iberian dry fermented sausages, human and pig feces were able to survive after 1.5 h of exposure at pH 2.5, where the number of final surviving bacteria ranges between 5.4 to 8.9 log cfu/g. In general, other food grad additives like glucose and prebiotics as well as cell protectants (exopolysaccharides, glycerol) enhance viability and tolerability of lactic acid bacteria under acid stressful conditions (Oh , 2000; Corcoran , 2005).

Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine.

Similarly, to exert probiotic sufficiency, desired strains should reach to the lower intestinal tract and maintain themselves overthere. Bile acids are synthesized in the liver from cholesterol and sent to the gall–bladder and secreted into the duodenum in the conjugated form (500-700 ml/day) after food intake by an individual (Hofmann and Roda, 1984). Bile plays an essential role in lipid digestion; it emulsifys and solubilizes lipids and functions as biological detergent. In the large intestine this acids suffer some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity. Conjugated and deconjugated bile acids show antimicrobial activity especially on *E. coli* subspecies, *Klebsiella* spp., and *Enterococcus* spp. in vitro. The deconjugated acid forms are more effective on gram positive bacteria (Dunne 1999, Çakır, 2003; Begley , 2006).

Bile salt hydrolases (BSHs) are generally intracellular, oxygen-insensitive enzymes that catalyze the hydrolysis of bile salts. Hydrolysis of bile salts is mediated by various genera of the intestinal microflora, including *Clostridium*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Kawamoto, 1989; Lundeen and Savage, 1990; Christiaens , 1992; Gopal , 1996; Grill , 2000; Franz , 2001). A number of BSHs have been identified and characterized in probiotic bacteria, and the ability of probiotic strains has often been included among the criteria for probiotic strain selection. Bile tolerance of probiotic bacteria can be investigated by incubating them for 24 hrs in a suitable medium containing different concentrations of bile extracts and monitoring cell viability and pH before and after incubation (Goktepe , 2006). This assay was used by several authors to assess the bile resistance of potential or already commercialized probiotic lactobacilli. All these studies reported a growth delay of lactobacilli in the presence of oxgall that was strain- and not species-dependent. It has been hypothesized that deconjugation of bile salts is a detoxification mechanism and BSH enzymes play a role in bile tolerance of probiotic organisms in the GIT (Savage, 1992). Both conjugated and deconjugated bile acids have been determined to inhibit the growth of *Klebsiella* spp., *Enterococcus* spp. and *Escherichia coli* strains in vitro. However, deconjugated forms of bile acids were found to be more inhibitory against Gram-positive than Gram-negative bacteria (Stewart , 1986). Studies by De Smet (1995) suggested that deconjugation of bile

acids decreases their solubility and thus diminishes the detergent's activity and makes it less toxic to bacteria in the intestine. It was assumed that the conjugated form of the bile salts exhibits toxicity by causing intracellular acidification through the same mechanism as organic acid. Another hypothesis states that certain Clostridium spp. utilize the amino acid taurine as an electron acceptor and have demonstrated improved growth rates in the presence of taurine and taurine-conjugated bile salts (Moser and Savage, 2001). However, taurine or taurine conjugates did not affect the growth of *Lactobacillus* spp. tested (Tannock 1989). Cholic acid was found to accumulate in *lactobacillus* cells by means of a transmembrane proton gradient (Kurdi , 2000). Whereas studies by Boever (2000) reported that cholic acid was highly deleterious for the viability of lactobacilli. It has also been suggested that the BSH enzymes are detergent shock proteins (Adamowicz , 1991) that protects the lactobacilli from its toxic effects and may have a competitive advantage over the non-BSH producing bacteria. However, studies of Moser and Savage (2001) reported that deconjugation and resistance are unrelated activities. Lastly, studies done by Gopal (1996) showed no relationship between the ability of 6 strains of L. acidophilus and 8 strains of *Bifidobacterium* spp. to grow in bile (0.3% oxgall) and their ability to hydrolyze bile salts (glycocholic acid or taurocholic acid). A link between bile salt hydrolysis and bile tolerance has been provided by the studies conducted on wild-type and bsh mutant pairs of *Lactobacillus plantarum*, *Lactobacillus amylovorus* and *Listeria monocytogenes*. Results showed that mutant cells were significantly more sensitive to bile and bile salts and displayed decreased growth rates in the presence of bile salts (De Begly , 2006).

1.5.2 *Antimicrobial Activity*

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens (Klaenhammer, 1999). Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Çakır, 2003; Quwehand and Vesterlund 2004). There are some researchs on showing that different species produce different antimicrobial substances. Here are some examples of these substances: *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance reuterin; subspecies of *Lactococcus lactis* produce a class I bacteriocin, nisin A; *Enterococcus feacalis* DS16 produces a class I bacteriocin cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin plantaricin S; *Lactobacillus acidophilus* produces a class III bacteriocin acidophilucin A (Quwehand and Vesterlund 2004). Production of bacteriocins is highly affected by the factors of the species of microorganisms, ingredients and pH of medium, incubation temperature and time.

Probiotics can balance intestinal bacteria by producing organic acid, bacteriocins, and antimicrobial peptides. This may lead to a competitive displacement of intestinal pathogens, the engagement of cell membrane receptors, which activate signaling events leading to cytokine synthesis, including interferons, and cell resistance to viral attack.

LAB produces lactic acid as the major metabolic end-product of sugar fermentation. Besides exerting its activity through lowering the pH and through its undissociated form, lactic acid is also known to function as a permeabilizer of the Gram-negative bacterial outer membrane, allowing other compounds to act synergistically with lactic acid (Niku-Paavola , 1999; Alakomi, 2000). Non-dissociated, or free, organic acids (RCOOH) can penetrate the bacterial cell wall, and thus enter the bacterial cytoplasm where they are exposed to a pH value near to neutrality (7.85 ± 0.05 for *E. coli*) and subsequently dissociate, releasing H^+ and anions (A-). As a result of accumulating protons, the internal pH decreases. In case of non-pH sensitive bacteria under low internal pH conditions, the organic acids remain in the free acid form and thus exit the bacteria. This process forms equilibrium between internal and external pH and protects the bacteria from detrimental effects of pH fluctuations (Lambert and Stratford, 1999; Gauthier, 2002). A specific mechanism (H^+-ATPase pump) in pH-sensitive bacteria acts to bring the pH inside the bacteria back to a normal level, as they can not tolerate a large spread between the internal and the external pH. This process consumes energy and eventually stops the growth of the bacteria or even kills it. A lower cytoplasmic pH inhibits glycolysis, prevents active transport, and also interferes with signal transduction. Furthermore, the anionic (A-) part of the acid cannot diffuse freely through the cell wall, and thus accumulates inside the bacterial cell. Accumulation of anions leads to internal osmotic problems for the bacteria (Lambert and Stratford, 1999).

Some LAB produce hydrogen peroxide (H_2O_2) under aerobic growth conditions and because of the lack of cellular catalase, pseudocatalase, or peroxidase, they release it into the environment to protect themselves from its antimicrobial action. It is a strong oxidizing agent and can oxidize the –SH group of membrane proteins of Gram-negative bacteria, which are especially susceptible (De Ray, 2004). *Lactobacillus gasseri* CRL1421 and *L. gasseri* CRL1412 were found to produce H_2O_2 and lactic acid *in vitro*, which reportedly reduced the viability of *Staphylococcus aureus* by 4 log units, after 6 h of incubation (Otero and Macias, 2006).

1.6 *Health benefits of probiotics*

A wide variety of potential beneficial health effects have been attributed to probiotics. Claimed effects range from the alleviation of constipation to the prevention of major life-threatening diseases such as inflammatory bowel disease, cancer, and cardiovascular incidents. Some of these claims, such as the effects of

probiotics on the shortening of intestinal transit time or the relief from lactose maldigestion, are considered well-established, while others, such as cancer prevention or the effect on blood cholesterol levels, need further scientific backup (Leroy 2008). The mechanisms of action may vary from one probiotic strain to another and are, in most cases, probably a combination of activities, thus making the investigation of the responsible mechanisms a very difficult and complex task. In general, three levels of action can be distinguished: probiotics can influence human health by interacting with other microorganisms present on the site of action, by strengthening mucosal barriers, and by affecting the immune system of the host (Leroy 2008).

Many probiotic mechanisms have been elucidated. Some are directed at pathogenic microorganisms that inhabit the gut, including competitive exclusion of bacteria and bacteriocidal activity. Others are directed at either the host epithelium or immune cells, including modulation of barrier function, innate and adaptive immunity, inflammatory signalling, and providing nutrition to the host through promotion of digestive capabilities and enhancing nutrient uptake (Corr , 2009; Yan and Polk, 2010). These mechanisms can involve the live bacterium, cell structure epitopes, nucleic acid components, or secreted factors. The mechanisms by which probiotics antagonize pathogenic infections and modulate cell signalling are further described below.

Probiotics are thought to work largely through direct or indirect effects on the gut microbiota and environment and/or on host function. A probiotic is a live micro-organism is consumed, in a range of dosages, spanning from ~10^8 to 10^{12} cells/day, depending on the product. This large number of microbes has the potential for a greater impact in the upper GI tract where lower densities of micro-organisms are found, but is also thought to impact the colon. Probiotics act on and interact with the host by two main modes of action, or a combination of actions (Figure |2): Impact of microorganisms or their metabolites/enzymes on the host's GI tract and its microbiota; and Interaction with the host's cells and immune system (O'Toole and Cooney 2008).

1.7 *GI tract and its microbiota*

As noted, bifidobacteria and lactobacilli preferentially ferment carbohydrates that escape digestion, resulting in a reduced pH. Bifidobacteria can ferment fructans because they have an enzyme, β-fructofuranosidase, that other bacteria either lack or have present at a lower activity, thus giving them a competitive advantage when exposed to fructans in the human gut. Similarly, the presence of β-galactosidase in lactobacilli or streptococci exerts a competitive advantage in GOS fermentation. The metabolism of prebiotic fructans by bifidobacteria yields mainly the acidic compounds acetate and lactate. Cross-feeding of these fermentation products to other species gives rise to butyrate and propionate. Butyrate and propionate are also formed from the direct fermentation of other dietary carbohydrates (Figure 02) (O'Toole and Cooney, 2008).

The benefits of a lower pH in the colon are that it encourages the multiplication and survival of commensal organisms that prefer acidic conditions and generally inhibits the ability of some pathogens to adhere, grow, translocate across the epithelium or colonise the GI tract. Furthermore, butyrate has long been known, from *in vitro* studies on fermentable dietary fibres, to enhance mucosal cell differentiation and this may also promote the barrier function of the epithelium (Blaut and Clavel, 2007; Roberfroid 2010).

Saccharolytic fermentation concomitantly reduces the potentially adverse effects of protein fermentation and other processes, which give rise to nitrogen and sulphur-containing compounds such as ammonia, N-nitroso- and azo- compounds as well as sulphides. Some probiotics may improve the barrier function of the mucus layer or epithelial cells. Evidence from cell culture studies suggests that an increase in the production of mucins may result from an enhancement of gene expression in the mucus-producing Goblet cells that line the GI tract. Increasing the mucous layer helps protect the epithelial cells from potential pathogen translocation and may enhance the clearance of pathogens from the GI tract (Roberfroid 2010).

Probiotics may also enhance the ability of specialised Paneth cells in the small intestine to produce the antibacterial peptides known as defensins. This hypothesis is supported by *in vitro* studies, using intestinal epithelial (e.g. Caco-2) cells grown in tissue culture, that have shown that certain probiotics can stimulate human β-defensin mRNA expression and peptide secretion (Ng , 2009). In vitro studies suggest that probiotics and prebiotics may affect the barrier function of the epithelium itself by enhancing the resistance of tight junctions, possibly via an effect on tight junction proteins (e.g. occludins and claudins).

Increased expression of genes encoding tight junction proteins has been shown in a study conducted in human volunteers administered a specific *Lactobacillus* strain. Animal and *in vitro* studies have found that specific probiotics can compete with pathogens for receptor sites on epithelial cells or in the mucous layer, thereby preventing pathogens from adhering or translocating (Sherman , 2005; Ohland and MacNaughton, 2010).

In contrast, other probiotics may directly bind to the pathogen, thus reducing its ability to colonise the intestine. There is good evidence from studies on mice that feeding on certain probiotic strains can greatly reduce the ability of pathogens such as *S. typhimurium* and pathogenic *E. coli* to translocate and invade the liver and spleen. In vitro studies showed that the same strains compete with the ability of pathogens to adhere to cells. Influence on pathogen translocation in infected animal models has also been shown for some prebiotics (Mattar , 2002; Mack , 2003).

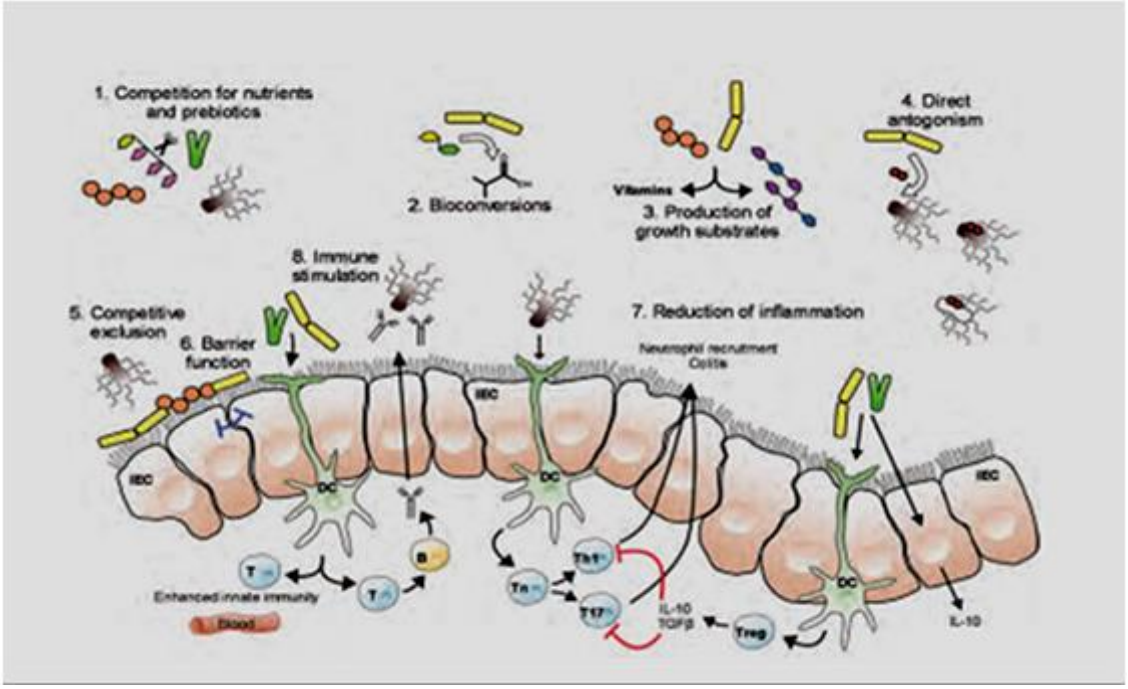


Figure 2: Diagram illustrating potential or known mechanisms of probiotics action. These mechanisms include (1) competition for dietary ingredients as growth substrates, (2) bioconversion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, for other bacteria, (4) direct antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colonisation and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC, epithelial cells; DC, dendritic cells; T, T cells. (O'Toole and Cooney, 2008).

1.8 *Cross-talk of probiotics with the host*

The most complex of the postulated mechanisms by which probiotics and stimulated endogenous microbes may act is the interaction with the GI immune cells and lymphoid tissue to modulate the immune and inflammatory responses of the host, which might lead to the potential for an impact beyond the gut (Figure 03). The mammalian immune system is generally considered to consist of two major arms: the innate (or non-specific immediate) immune response and the acquired (or specific adaptive) immune response. Both parts of the immune system are extremely complex and involve cells (cellular immunity) and other components secreted into the blood (e.g. antibodies and cytokines). The two arms work together to protect the host from pathogens (bacteria, viruses, fungi), other foreign materials (antigens) and also from tumour cells arising in the host (ILSI, 2011). Through so-called bacterial–epithelial cell "cross-talk", it seems that ingested and endogenous microbes can impact both the innate and the adaptive responses of the host immune system. The interaction between microbial cells (commensal, probiotic or pathogen) and host cells is mediated by the interaction with specific receptors such as Toll-like receptors (TLR) that are associated with cells lining the mammalian GI tract. The activation of these receptors initiates a cascade of concerted immune signals leading to different responses.

For example, the response can ensure a balanced maturation of T cells (Th1 versus Th2) and T-regulatory cells, which allows an appropriate response to potential pathogens and food antigens. An inappropriate T cell response is thought to be one of the features of allergic conditions, as mentioned previously. Further, activation of the immune pathways can also result in B cell differentiation and production of protective antibodies, such as IgA secreted into the intestinal lumen (Abreu 2003; Bäckhed and Hornef 2003, Takeda 2003; Vinderola 2005). Along the same lines, the ingestion of specific probiotic strains or prebiotics in human and animal studies has been found to stimulate an increase in the anti-inflammatory cytokines, such as IL-10 and TGF-β, and a decrease in the expression of pro-inflammatory cytokines, such as TNF-α and IFN-γ.

It is proposed that these changes in cytokine balance could be a mechanism by which prebiotics and probiotics may be able to mitigate chronic intestinal inflammation. The activity of phagocytic cells (neutrophils and macrophages) and natural killer (NK) cells (non-T non-B lymphocytes), which are part of the innate immune response, is also modulated in animals and humans by various probiotics and to some extent by prebiotics or synbiotics. In addition, animal studies have suggested that the so-called G-protein receptors in certain white blood cells may act as receptors for SCFA, increased levels of which result from the ingestion of prebiotics, thus opening up the possibility of alternative mechanisms impacting the immune system (Rhee 2005; Pedersen 2005).

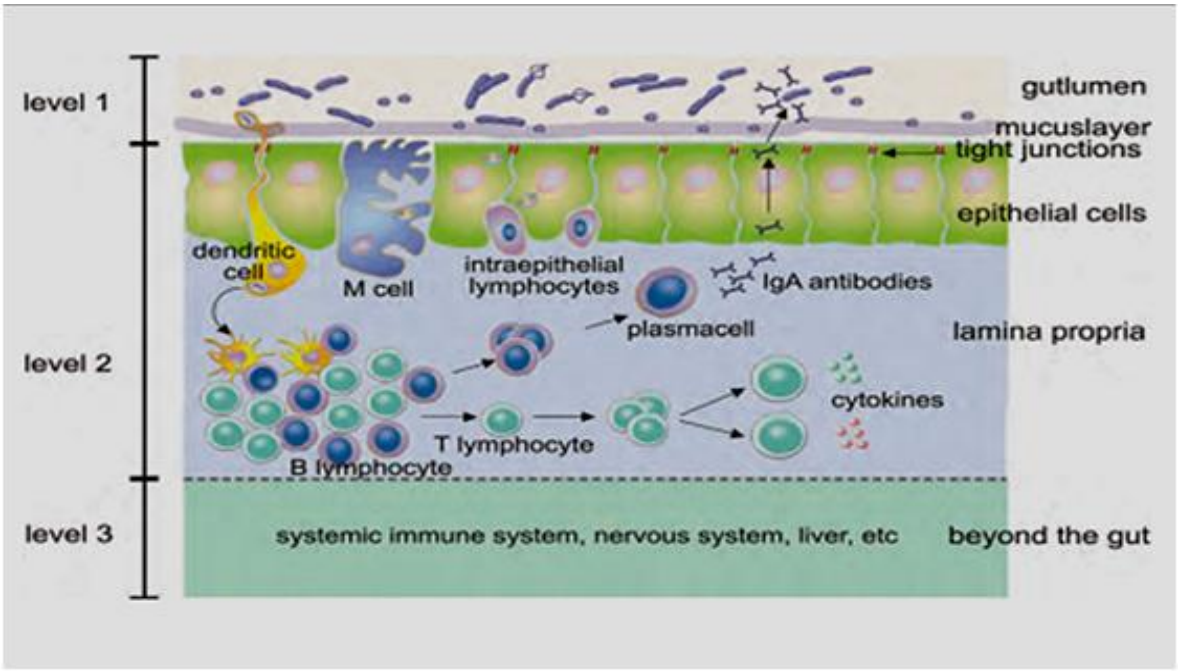
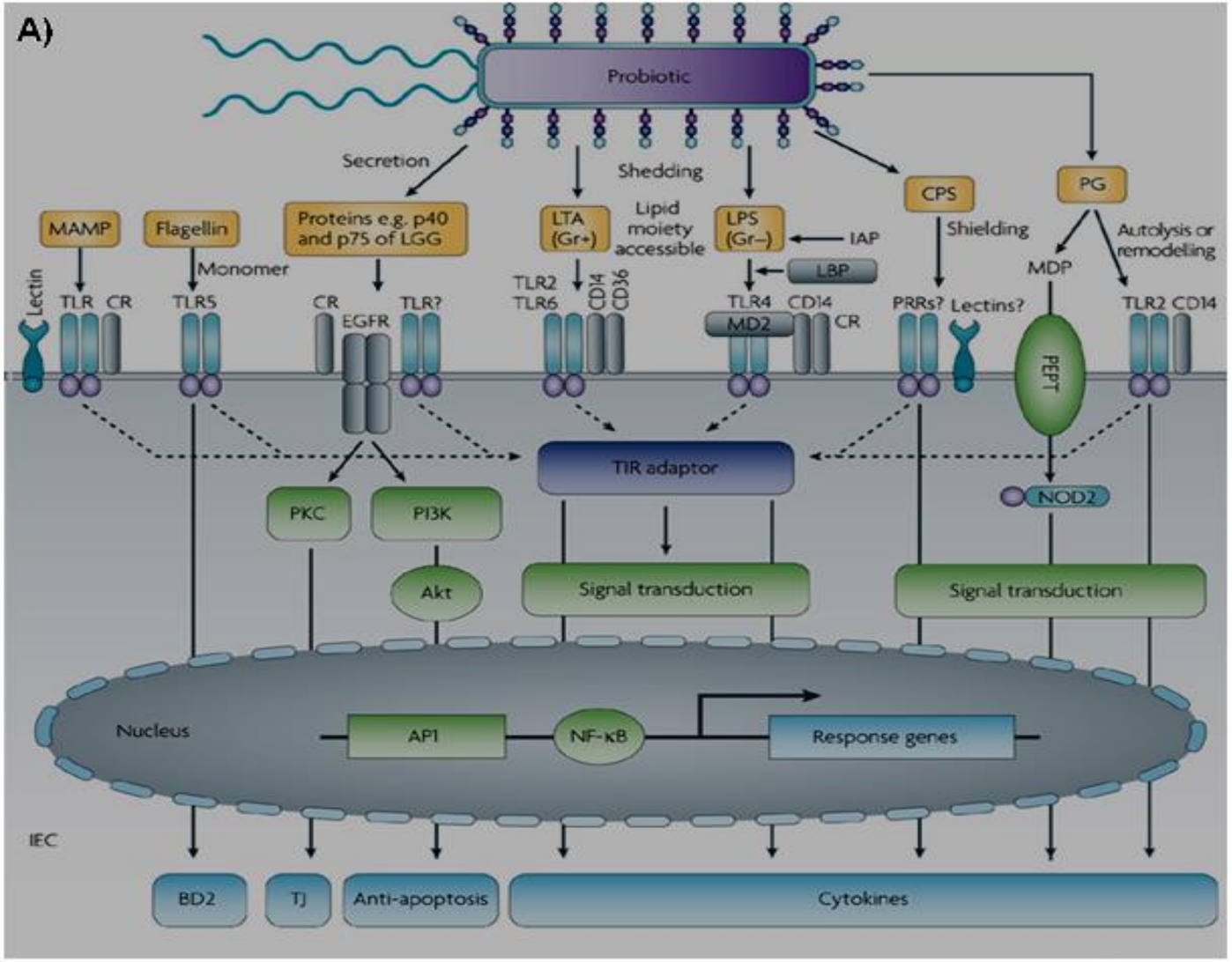


Figure 3: The three levels of action of a probiotic. Probiotic bacteria can interfere with the growth or survival of pathogenic micro-organisms in the gut lumen (level 1). Probiotic bacteria can improve the mucosal barrier function and mucosal immune system (level 2) and, beyond the gut, have an effect on the systemic immune system, as well as other cell and organ systems such as liver and brain (level 3). (Rijkers, 2010).


A)
Probiotic
Secretion
Shedding
CPS
PG
MAMP
Flagellin
Proteins e.g. p40 and p75 of LGG
LTA (Gr+)
Lipid moiety accessible
LPS (Gr–)
IAP
LBP
Shielding
Autolysis or remodelling
MDP
Monomer
Lectin
TLR CR
TLR5
CR EGFR
TLR?
TLR2 TLR6
CD14 CD36
TLR4 CD14
MD2
CR
PRRs?
Lectins?
PEPT
TLR2 CD14
TIR adaptor
PKC
PI3K
NOD2
Akt
Signal transduction
Signal transduction
Nucleus
AP1
NF-κB
Response genes
IEC
BD2
TJ
Anti-apoptosis
Cytokines

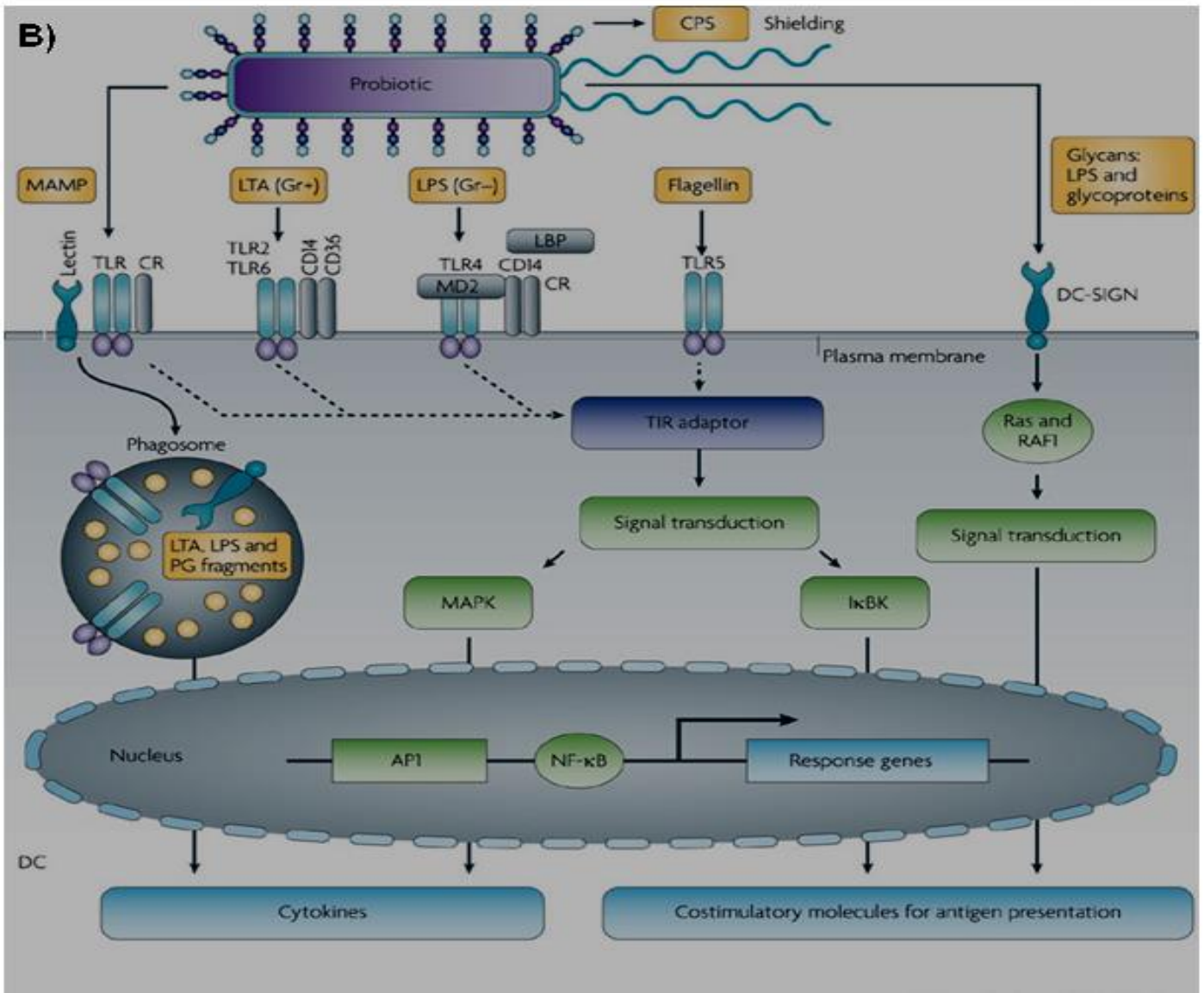

B)
CPS
Shielding
Probiotic
Glycans: LPS and glycoproteins
MAMP
LTA (Gr+)
LPS (Gr–)
Flagellin
LBP
Lectin
TLR CR
TLR2 TLR6
CD14 CD36
TLR4 CD14
MD2
CR
TLR5
DC-SIGN
Plasma membrane
TIR adaptor
Ras and RAF1
Phagosome
LTA, LPS and PG fragments
Signal transduction
Signal transduction
MAPK
IκBK
Nucleus
AP1
NF-κB
Response genes
DC
Cytokines
Costimulatory molecules for antigen presentation

Figure 4:

A) Probiotic MAMP–PRR interactions in IECs and associated signalling events. Probiotic bacteria can interact with intestinal epithelial cells (IECs) using various surface molecules. Demonstrated effects of these interactions include: flagellin-mediated induction of human β-defensin 2 (BD2; also known as DEFB4) by *Escherichia coli* Nissle 1917, *Lactobacillus rhamnosus* GG p40- and p75 (cell wall-associated glycoside hydrolase)-mediated anti-apoptotic and tight junction (TJ)-protecting effects; and induction of cytokines by lipoteichoic acid (LTA)–Toll-like receptor 2 (TLR2), lipopolysaccharide (LPS)–TLR4 and peptidoglycan (PG)– nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (see main text for details). The accessibility of these microorganism-associated molecular patterns (MAMPs) for the host pattern recognition receptors (PRRs) varies considerably. For example: flagellin needs to interact as monomers with TLR5; p40 and p75 can mediate their effects after secretion; LPS and LTA need to be shed from the bacterial cell wall for the lipid moiety to become accessible; and PG needs to be hydrolysed (autolysis or remodelling). In addition, various co-receptors (CRs) that are associated with PRRs in lipid rafts can fine-tune MAMP–PRR signalling. Moreover, IECs have developed specific features for MAMP–PRR signalling. In healthy subjects, IECs are generally hyporesponsive for LTA and LPS from gut microorganisms, owing to several control mechanisms, including downregulation of TLR2 and TLR4 expression. The apical intestine alkaline phosphatase (IAP) enzyme reduces LPS–TLR4 signalling by detoxifying LPS. PG interacts only with NOD2 after its ligand, muramyl dipeptide (MDP), is taken up by the apical peptide transporter PEPT1 (also known as SLC15A1). The interaction of probiotic cell wall-associated polysaccharide (CPS) molecules with PRRs or associated lectin-like co-receptors in IECs is not well understood, but some, such as the K5 capsule of *E. coli* Nissle 1917, have a documented cytokine-inducing capacity. CPS molecules could also modulate the interaction of other MAMPs with their respective PRRs by shielding effects. EGFR, epidermal growth factor receptor; Gr+, Gram-positive bacteria; Gr–, Gram-negative bacteria; LBP, LPS-binding protein; LGG, *Lactobacillus rhamnosus* GG; MD2, also known as LY96; NF-κB, nuclear factor- κB; PI3K, phosphoinositol 3-kinase; PKC, protein kinase C; TIR, Toll/interleukin-1 receptor.

B) Probiotic MAMP–PRR interactions in DCs and associated signaling events. Probiotic bacteria can interact with dendritic cells (DCs) using various surface molecules. Demonstrated effects of interactions include lipoteichoic acid (LTA)–Toll-like receptor 2 (TLR2)-mediated and lipopolysaccharide (LPS)–TLR4-mediated induction of cytokines and co-stimulatory molecules for antigen presentation that can modulate T cell polarization. These interactions seem to be promoted by ingestion of the bacteria through phagocytosis and digestion in phagolysosomes, so that the interacting microorganism-associated molecular patterns (MAMPs) such as lipid A from LPS or the two acyl chains from LTA become accessible for pattern recognition receptor (PRR) recognition. In addition, the putative glycoprotein S layer protein A (SlpA) of *Lactobacillus plantarum* was shown to interact with DCs through the C type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). DC-SIGN is mainly a phagocytotic receptor, promoting uptake of the bacteria in phagolysosomes, but can also induce signalling that involves the small G protein Ras and the kinase RAF1, which mediates interaction with nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK). Cell wall-associated polysaccharide (CPS) could modulate these interactions by forming a shield that impedes phagocytosis. CR, co-receptor; Gr+, Gram-positive bacteria; Gr–, Gram-negative bacteria; IκBK, inhibitor of NF-κB kinase; LBP, LPS-binding protein; MD2, also known as LY96; PG, peptodiglycan; TIR, Toll/interleukin-1 receptor. Reproduced from *Lebeer S, Vanderleyden J, De Keersmaecker SCJ. 2010. Nature Reviews Microbiology 8: 171-184.*

Although studies in humans have found changes in biomarkers such as cytokine levels and changes in the number and activity of immune cells, it is nevertheless of prime importance to have studies in human subjects that also measure clinical outcomes. Clinical measures, such as a reduced incidence of infection or enhanced immune response to a vaccine, can then be linked to measures of humoral or cellular immune biomarkers. Even though results from animal studies cannot necessarily be extrapolated to humans, in vivo studies in animal models represent a valuable means of understanding the complex signalling cascade underlying a protective immune response (Lebeer 2010; ILSI Europe 2011).

1.9 *Origine and safety of probiotics*

An old dogma of probiotic selection has been that the probiotic strains should be of "human origin". One may argue that from evolutionary point of view, describing bacteria to be of human origin does not make much sense at all. The requirement for probiotics to be of human origin relates actually to the isolation of the strain rather than the "origin" itself. Usually, the strains claimed to be "of human origin" have been isolated from faecal samples of healthy human subjects, and have therefore been considered to be "part of normal healthy human gut microbiota". In reality the recovery of a strain from a faecal sample does not necessarily mean that this strain is part of the normal microbiota of this individual, since microbes passing the GI tract transiently can also be recovered from the faecal samples (Forssten , 2011). In practice it is impossible to know the actual origin of the probiotic strains, regardless of whether they have been isolated from faecal samples, fermented dairy products or any other source for that matter. Isolation of a strain from faeces of a healthy individual is also not a guarantee of the safety of the strain—such a sample will also always contain commensal microbes which can act as opportunistic pathogens, or even low levels of true pathogens, which are pres- ent in the individual at sub-clinical levels. Therefore, it has been recommend that instead of concentrating on the first point of isolation, the selection processes for new potential probiotic strains should mainly focus on the functional properties of the probiotic strains rather than the "origin" (Forssten , 2011; Ouwehand and Lahtinen 2008).

As viable, probiotic bacteria have to be consumed in large quantities, over an extended period of time, to exert beneficial effects; the issue of the safety of these microorganisms is of primary concern (Leroy 2008). Until now, reports of a harmful effect of these microbes to the host are rare. However, many species of the genera *Lactobacillus, Leuconostoc, Pediococcus, Enterococcus*, and *Bifidobacterium* were isolated frequently from various types of infective lesions. According to Gasser (1994), *L. rhamnosus, L. acidophilus, L. plantarum, L. casei, Lactobacillus paracasei, Lactobacillus salivarius, Lactobacillus lactis*, and *Leuconostoc mesenteroides* are some examples of probiotic bacteria isolated from bacterial

endocarditis; *L. rhamnosus, L. plantarum, Leuc. mesenteroides, Pediococcus acidilactici, Bifidobacterium eriksonii,* and *Bifidobacterium adolescentis* have been isolated from bloodstream infections and many have been isolated from local infections. Although minor side effects of the use of probiotics have been reported, infections with probiotic bacteria occur and invariably only in immunocompromised patients or those with intestinal bleeding (Leroy 2008).

An issue of concern regarding the use of probiotics is the presence of chromosomal, transposon, or plasmid-located antibiotic resistance genes amongst the probiotic microorganisms. At this moment, insufficient information is available on situations in which these genetic elements could be mobilised, and it is not known if situations could arise where this would become a clinical problem (Leroy 2008). When dealing with the selection of probiotic strains, the FAO/WHO Consultancy recommends that probiotic microorganisms should not harbor transmissible drug resistance genes encoding resistance to clinically used drugs (FAO/WHO, 2002). For the assessment of the safety of probiotic microorganisms and products, FAO/WHO has formulated guidelines, recommending that probiotic strains should be evaluated for a number of parameters, including antibiotic susceptibility patterns, toxin production, metabolic and haemolytic activities, and infectivity in immunocompromised animals (FAO/WHO, 2002). In vitro safety screenings of probiotics may include, among others, antibiotic resistance assays, screenings for virulence factors, resistance to host defence mechanisms and induction of haemolysis. Several different animal models have been utilized in the safety assessment of probiotics. These include models of immunodeficiency, endocarditis, colitis and liver injury. In some cases even acute toxicity of probiotics has been assessed. Last but not least, also clinical intervention trials have yielded evidence on the safety of probiotics for human consumption (Forssten , 2011).

Chapter 02

Bio-Control of Helicobacter pylori

2. Management of *Helicobacter pylori* Infection by Antibiotics *and* Probiotics.

2.1 *General aspects of H. pylori infection*

Microbial persistence in the presence of a host response by a colonized host is a seeming paradox. Yet in human biology there are prominent examples of this phenomenon, including HIV, *Plasmodium*, and *Mycobacterium tuberculosis* infections. Well-adapted microbes have evolved ways not only to circumvent these mechanisms but also to utilize host responses to their own advantage. Such a phenomenon has been postulated for the highly prevalent human gastric bacterium, *Helicobacter pylori*, which induces a host response in virtually all carriers but in a subset can augment the risk of peptic ulceration and distal gastric cancers (Blaser and Kirschner, 1999).

Helicobacter pylori is a gram-negative, curved or slightly spiral, microaerophilic, slow-growing organism. Its most characteristic enzyme is a potent multi-subunit urease. *H. pylori* is motile and possess four to six sheathed polar flagella (Khalifa , 2010). The organism appears to have a limited metabolic and biosynthetic capacity. These characteristics are consistent with those of an organism that colonizes a restricted ecological niche. This bacterium is specialized in the colonization of the human stomach, a unique ecological niche characterized by very acidic pH, a condition lethal for most microbes. *H. pylori* is so well adapted to this unfriendly environment that, after the first infection, which usually occurs early in life, it establishes a life-long chronic infection. The selection of a niche with no competition and the ability to establish a chronic infection make *H. pylori* one of the most successful human bacterial parasites, which colonizes more than half of the human population (Montecucco and Rappuoli, 2001). This bacterium is the only formally recognized definitive bacterial carcinogen for humans and is estimated to be responsible for 5.5% of all human cancer cases or approximately 989 598 gastric cancer cases per year. It has also become a paradigm for a bacterium that causes chronic infections, and its mode of action as a pathogen has been termed ‘slow’ or ‘stealth’ (Suerbaum and Josenhans, 2007; Yamaoka, 2012). Little is known about the relationships between the genetic diversity in *H. pylori* and the survival of the bacteria in their individual human hosts. However, it has been assumed that, adaptation processes (chronic lifestyle, persistence, host-specificity, and the lack of a natural reservoir outside humans) that take place in the context of the host environment are responsible for bacterial diversification (Suerbaum and Josenhans, 2007).

Since the discovery of *H. pylori* by Marshall and Warren, a strong link has been established between *H. pylori* and a diverse spectrum of gastrointestinal diseases, including gastric and duodenal ulceration, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma, and non-Hodgkin's lymphoma of the stomach. As a result, the World Health Organization classified *H. pylori* as a class I carcinogen for gastric cancer and currently this organism is considered the most common etiologic agent of infection-related cancers, representing 5.5% of the global cancer burden. *H. pylori* inhabits the human stomach for decades and recent evidence now supports the tenet that *H. pylori* has coevolved with humans for tens of thousands of years, with genetic studies indicating that humans have been colonized with *H. pylori* for at least 58,000 years. Present in approximately half of the world's population, *H. pylori* remains the most common bacterial infection in humans. Although the majority of individuals infected with *H. pylori* remain asymptomatic throughout their life, essentially all develop chronic inflammation. Among infected individuals, approximately 10% develop peptic ulcer disease, 1–3% develops gastric adenocarcinoma, and less than 0.1% develops MALT (Noto and Peek, 2012).

2.2 *Incidence and distribution*

In the modern world, *H. pylori* infections are responsible for a heavy toll of morbidity and mortality as a consequence of ulcer disease, lymphoma of the mucosa-associated lymphoid tissue (MALT) and, the most dangerous complication of *H. pylori* infection, gastric adenocarcinoma (Suerbaum and Josenhans, 2007).

The prevalence of *H. pylori* shows large geographical variations. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages. The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Figure 05). Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood. While the prevalence of *H. pylori* infection in developing countries remains relatively constant, it is rapidly declining in the industrialized world. In developing countries, *H. pylori* infection rates rise rapidly in the first 5 years of life and remain constantly high thereafter, indicating that *H. pylori* is acquired early in childhood**.** However, in industrialized countries the prevalence of *H. pylori* infection is low early in childhood and slowly rises with increasing age. This increase results only to a small extent from *H. pylori* acquisition at later age. The incidence of new *H. pylori* infections among adults in the Western world is less than 0.5% per year; the higher prevalence of infection among the elderly thus reflects a birth cohort effect with higher infection rates in the past. The active elimination of *H. pylori* from the population and improved hygiene and housing conditions have resulted in a lower infection rate in children, which is reflected in the age distribution of this lifelong-colonizing bacterium.

2.2 *Natural reservoirs and transmission routes*

Traditionally, the human stomach has been viewed as an inhospitable environment for microorganisms because of acidic conditions and other antimicrobial factors. With the discovery of *H. pylori* and other gastric helicobacters, and subsequent insight into the mechanisms by which these organisms adapt to the gastric environment, the existence of a bacterial community adapted to this human niche became more plausible (Figure 06). *H. pylori* is known to resides in human stomach. Other possible extra gastric ecological niche for *H. pylori* being evaluated is the oral cavity. The bacterium has been detected in saliva, in the microbiota from the dorsum of the tongue, on the surface of oral ulcerations, oral neoplasia and in dental plaque. High rates of *H. pylori* detection in dental plaque and saliva suggest that the oral cavity might be an important reservoir of the bacterium which can lead to infection in the stomach. Kamada (2006) reported three cases of *H. pylori* related acute gastric mucosal lesions following dental treatment in all patients. Furthermore, dental plaque has been implicated as a possible source of gastric reinfection after apparently successful gastric eradication as *H. pylori* eradication therapy successfully removes the bacterium from stomach but not from dental plaque (Chaudhry 2009).

Environmental or animal reservoirs were investigated as sources of *H. pylori* infection. Food, animals, and water sources have been suggested as reservoirs outside the human gastrointestinal tract, and *H. pylori* or its DNA was detected in each of these sources (Table 04). However, there is no definitive evidence that they are natural or primary vehicles of transmission (Khalifa 2010).

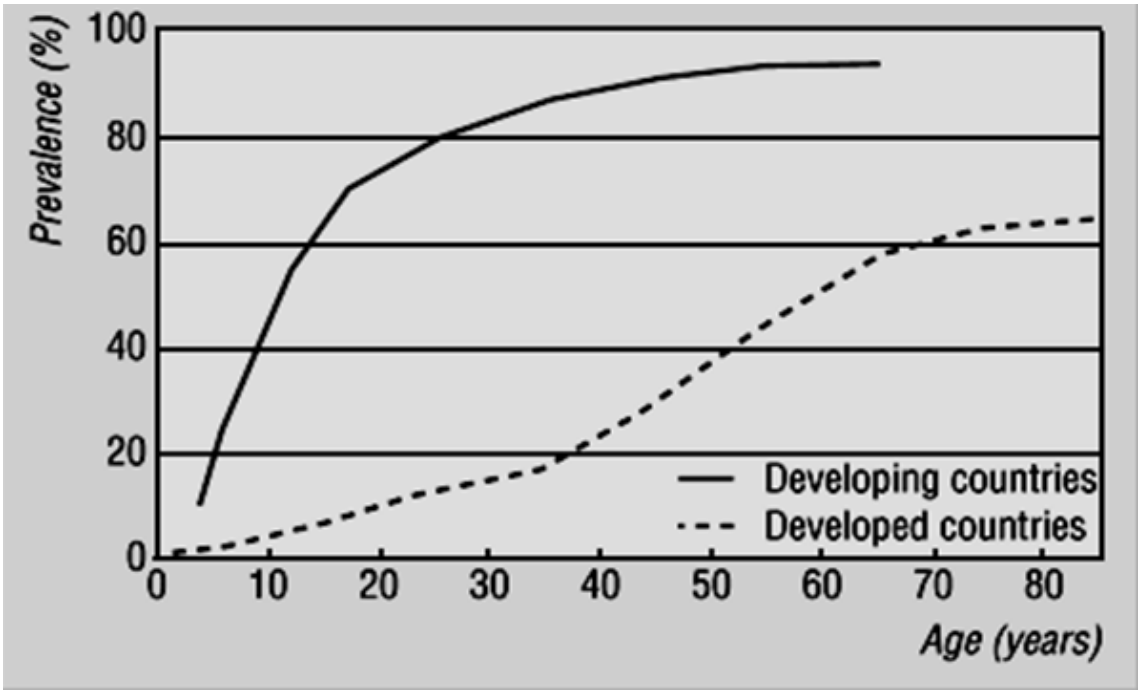


Figure 05: Prevalence of *H. pylori* in human populations worldwide (developing and developed countries) (Logan and Walker, 2001).

Approximately 50% of the world's population is affected by gastric *H. pylori* infection. Infection is significantly more prevalent in developing countries where reported prevalence in adult population is around 90% as compared to less than 40% in developed nations (Fig.06). Asians carry higher prevalence of *H. pylori* infection with large inter-country variation. Infection is more frequent in less developed countries like Pakistan, India and Bangladesh, as compared to Japan and China. *H. pylori* infection is very common in Pakistan with infection rates reported to be as high as 90% in adult population. The exposure rate in children is around 33% with infection rates of 67% in infants and 30% in children under fifteen years of age (Chaudhry, 2009). Apart from human gastric mucosa, the organism has also been found in ectopic gastric mucosa such as in Barrett's oesophagus, and ectopic gastric mucosa in a Meckel's diverticulum. However, humans appear to be the only natural host for *H. pylori*. A number of animals (pigs, sheep and cats) have been suspected of harbouring *H. pylori* in their stomachs, but the spiral organisms identified by more discriminating molecular techniques often proved to be *Helicobacter* species that were closely related to, but different from, *H. pylori* (Luman, 2002).

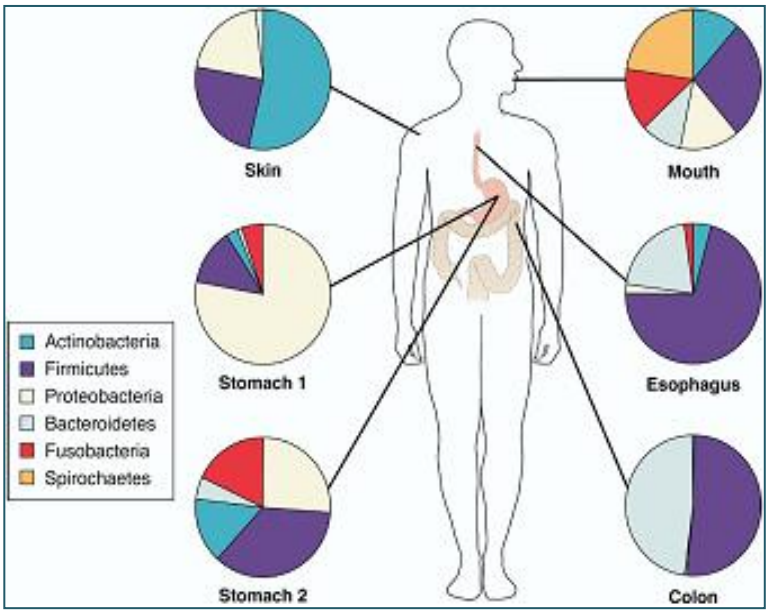


Figure 06: Major bacterial phyla in the stomach and at diverse anatomical sites. Stomach 1 depicts a stomach in which *H. pylori* is detected by conventional methods, and Stomach 2 depicts a stomach in which *H. pylori* is not detected (Cover and Blaser, 2009**).**

The route of transmission of *H. pylori* is not completely understood. The only known reservoir of *H. pylori* is the human stomach and since *H. pylori* appears to have a narrow host range; new infections are thought to occur as a consequence of direct human-to-human transmission or environmental contamination. There is evidence supporting a gastro–oral, oral–oral and faecal–oral transmission, but no conclusive data addressing the predominance of transmission via any of these routes exist. The minimum *H. pylori* infectious dose in non colonized rhesus monkey is 10^4 bacteria, but the bacterial load for secondary infection after antibiotic therapy decrease by a 10- to 100-fold. In humans the minimum dose have not been determined, but in human volunteers a dose ranging study (from 10^4 to 10^{10}) suggested that 10^5 colony forming units might be near the minimal infectious dose (Vale and Vítor, 2010).

2.3.1 *Person-to-person transmission*

Person-to-person transmission can be subdivided in two main categories: vertical and horizontal transmission. The vertical transmission is infection spread from ascendants to descendents within the same family, while horizontal transmission involves contact with individuals outside the family but does not exclude environment contamination (Schwarz , 2008). Type of strains circulating within-family members are often similar, namely between mother and child. In populations with low *H. pylori* prevalence, the infected mother is likely to be the primary source for infant *H. pylori* infection (Weyermann , 2009). The presence of common strains within the same family does not exclude acquisition of *H. pylori* from a common (external) source. *H. pylori* infection may be acquired by more diverse routes than previously expected (Raymond , 2008). The person-to-person transmission may occur by three possible pathways: the gastro–oral, the oral–oral and the faecal–oral.

2.3.2 *Gastro–oral transmission*

H. pylori is acquired in early life and the vomiting of achlorhydric mucus may serve as a vehicle for transmission. As reported by Axon (1995), the transmission route could be by gastric juice, especially as a result of epidemic vomiting in childhood. *H. pylori* appears to survive outside the human body in unbuffered gastric juice. Culture of *H. pylori* was possible in 62% of the 21 patients following 2 h of gastric juice collection, 42% after 6 h and 10% after 24 h (Galal , 1997). The culture of *H. pylori* from vomitus has reported in other studies (Leung , 1999; Luzza , 2000; Parsonnet , 1999; Perry , 2006). *H. pylori* was often present in high quantities in vomitus, with as many as 3 10^4 CFU/mL of sample, capable of being cultured from air samples collected after vomiting, but not before. However, the short duration of the contamination and the limited dispersion of organisms (less than 1.2 m) makes aerosol exposure an unlikely source of infection (Parsonnet , 1999). These results are favourable to the gastro– oral transmission, especially during childhood, coupled with poor hygiene practices, with the vomitus working as the putative vehicle of transmission (Vale and Vítor 2010).

2.3.3 *Oral–oral transmission*

Saliva is another possible source of *H. pylori* transmission, since the gastric flora can reach and colonize the mouth after regurgitation or vomiting. *H. pylori* DNA has been frequently amplified from saliva, subgingival biofilm and dental plaque. *H. pylori* has also been cultured directly from saliva. Therefore, the mouth might be a reservoir of *H. pylori*. However, the recovery of *H. pylori* does not seem to increase after vomiting and quantities of *H. pylori* in saliva tended to be low (Vale and Vítor, 2010). Furthermore, Kivi (2003) reported the presence of common strains infecting couples. Common strains with couples suggest person-to-person transmission or common source exposure within couples (Singh , 1999; Georgopoulos , 1996). These studies suggest that although saliva might work as a vehicle of transmission, the oral–oral transmission is not the main mode of transmission of *H. pylori*, at least in adults (Luman , 1996).

2.3.4 *Faecal–oral transmission*

H. pylori DNA has been frequently detected in human faeces (Vale and Vítor 2010). Attempts to culture *H. pylori* from faeces have had limited success (Dore , 2000; Falsafi , 2007; Liang and Redlinger, 2003) as the bacterium exists there predominantly in a nonculturable (coccoid) form (Kabir, 2001). Transmission via faecal contaminants is also supported by the occurrence of *H. pylori* infections among institutionalized young people during outbreaks of gastroenteritis (Laporte , 2004). Taken together, these findings support faecal–oral transmission in the transmission of *H. pylori*, especially when the hygienic conditions are poor.

Table 04 : *Helicobacter pylori* reservoirs (Khalifa , 2010).

Hypothesis	Evidence/Example studies
Food	
- Contaminated food prepared under unhygienic conditions is a probable mechanism for transmission.	A positive correlation was reported between prevalence of infection and consumption of food from street vendors in Peru.
- The daily amount of raw vegetables is a risk factor, which possibly implies a role for water too; - Sheep and cow milk can be vehicles for transmission.	In the Colombian Andes, frequent consumption of raw vegetables was associated with likelihood of infection. See below (under Animals)
Animals	
- Several animal species were suggested as *H. pylori* reservoirs.	H. pylori was isolated from: pigtailed monkeys, rhesus monkeys, cats , sheep, cockroaches, houseflies (but the housefly hypothesis was challenged).
- Working with animals may increase risk, and animal-to-human transmission is possible.	*H. pylori* was suggested as zoonotic, occupational infection to meat and abattoir workers. In Colombian Andes, children who had contact with sheep had higher prevalence odds. Dore stated that animals, especially sheep and dogs, could transmit *H. pylori* to humans (shepherds) in Sardinia
- *H. pylori* can be recovered from animal products. - Experimental animal colonization is possible.	*H. pylori* was recovered from sheep and cow milk. Bacillary forms of H. pylori were used to colonize germ-free piglets.
Water	
- Water contamination is a risk factor for *H. pylori* infection.	Many reports, mostly from developing countries, suggested contaminated water sources as risk factors. Examples include studies performed in Peru, Chile, and Kazakhstan.
- Water from running, municipal, and underground sources, as well as wastewater has been suggested as reservoir for *H. pylori.*	- Municipal water - Well water - Running water - Wastewater - *H. pylori* even survives in chlorinated water.
- *H. pylori* proteins and DNA can be detected in water.	- by immunological methods - by PCR - by hybridization methods
- Viable *H. pylori* can be detected and isolated from water.	- as individual cells - associated with biofilm - cultured

2.4 *Helicobacter pylori* colonization and persistence

The human stomach is far from a stable niche**.** Many pathogenic neutralophiles can survive in acidic environments by maintaining cytoplasmic pH at ~5.0. However, although they can survive in an acidic environment, they do not grow. Hence, they can transit but not colonize the stomach. Examples of such organisms are *Salmonella typhimurium*, *Vibrio cholera*, and pathogenic strains of *Escherichia coli* and *Yersinia eneterocolitica*. Although *H. pylori* is also a neutralophile, it is able not only to survive in but also to colonize (*i.e.*, grow within) the human stomach. Other *Helicobacter* species, such as *H. felis* or *H. mustelae* can colonize the cat and ferret stomach, respectively. Hence, gastric *Helicobacter* spp. have evolved specialized processes that allow maintenance of the pH of their cytoplasm at a level that enables the organisms to survive and grow. These properties of gastric *Helicobacter* spp. have been termed "acid acclimation" to differentiate them from the acid-resistance (acute) or acid-tolerance (chronic) mechanisms of neutralophiles in general (Sachs , 2005). According to which is believed, there are no bacteria, with the exception of the *Helicobacter* genus, that can establish long-term residence in the human gastric mucosa. In the acidic environment of the stomach, the ability of *H. pylori* to adapt is indispensable for its persistence. Van Amsterdam (2006) proposed two types of adaptation for *Helicobacter pylori* to its environment: short-term and long-term.

Helicobacter pylori chronically infects the human gastric mucosa prior to disease. Therefore, factors contributing to *H. pylori* colonization and persistence are inherently linked to *H. pylori*-related pathogenesis. Upon entrance of the gastric lumen, *H. pylori* has to cope with gastric acidity. It survives this acidity through its acid resistance: it then traverses the mucus layer to reach its niche close to the gastric epithelial cells. Chemotaxis, motility and adhesion are important processes in the colonization of the gastric epithelial cells. For long-term persistence in the human gastric mucosa, *H. pylori* also continuously requires nutrients from its host. This is achieved through degradation of the integrity of the mucus layer and the underlying gastric epithelial cells. Moreover, clearing of *H. pylori* is avoided by suppression of the host immune system, antigenic variation and antigenic mimicry (Van Amsterdam , 2006).

2.5 *Infection outcome and gastric diseases*

The infection by *H. pylori* causes a lifelong inflammation of the gastric mucosa, gastritis, in all infected individuals although usually clinically asymptomatic. The superficial gastritis progress towards chronic active gastritis characterized by infiltrating neutrophils, B cells, T cells, lymphocytes, macrophages and plasma cells. About 10-20% of the *H. pylori* infected individuals will develop peptic ulcer disease and 1-2% gastric cancer (Suerbaum and Josenhans, 2007) (Figure 07).

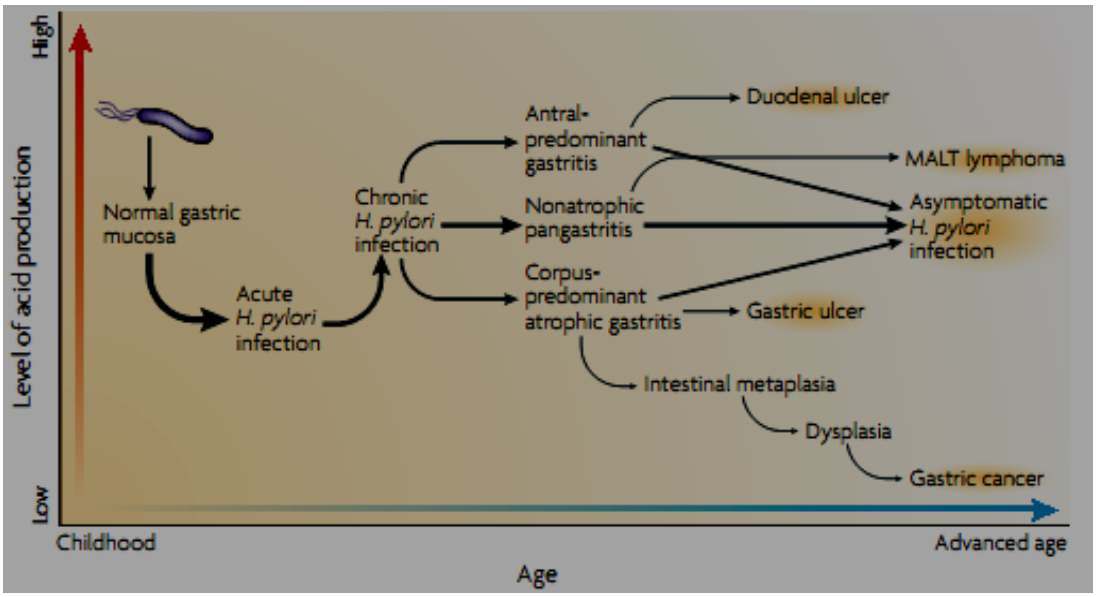


Figure 07: Natural progression of *H. pylori* infection. Infection usually occurs during childhood and causes symptomatic acute gastritis. Because the symptoms of acute gastritis are non-specific and transient, a diagnosis is rarely made at this stage. Acute infection transforms to chronic active gastritis in most patients and persists for decades or is life-long. The infection can take multiple courses. Most people that are infected with H. pylori will never develop symptomatic disease. 10–15% will develop ulcer disease (gastric or duodenal ulcer), approximately 1% will develop gastric adenocarcinoma, and a small group of patients will develop gastric MALT lymphoma (Suerbaum and Josenhans, 2007).

Peptic ulcer disease includes both gastric and duodenal ulcers, which are ulcers that develop in the corpus region and in the transition between the pylorus and duodenum, respectively (Figure 08). Gastric cancer is the fourth most common cancer worldwide with 934,000 cases each year; 490,000 of these cases are reportedly caused by *H. pylori* (IARC, 2008). *H. pylori* caused gastric cancer includes gastric adenocarcinomas and the more uncommon gastric mucosa-associated lymphoid tissue (MALT) lymphoma which affect B cells. About 80% of all gastric MALT lymphomas are caused by *H. pylori* and the majority will have a complete regression if the *H. pylori* is eradicated with antibiotics (Parsonnet 1994; Bayerdörffer 1995).

Not all *H. pylori* infected individuals will develop peptic ulcer disease or gastric cancer and why some infections progress into disease and others not is not completely understood. However, some factors are known to contribute to the clinical outcome i.e., the location of the *H. pylori* colonization in the stomach, hormonal changes, acid secretion, polarization of the T helper (Th) type of immune responses, virulence of the bacterial strain and life-style associated factors (Atherton 2006).

The location of the *H. pylori* infection in the stomach is dependent on the level of acid produced in the infected person. In a high-acid producing person, the infection will localize predominantly in the less acidic antrum. This antral-predominant infection and associated inflammation leads to even higher acid production levels because of the stimulation of parietal cells in the uninflamed corpus to produce more acid stimulated by the hormone gastrin. Hence, *H. pylori* infection established in the lower part of the stomach predisposes individuals to duodenal ulcer formation. In contrast, individuals with low acid production e.g., individuals with inhibition of parietal cell acid production by interleukin-1β (IL-1β) will develop severe hypochlorhydria and corpus predominant gastritis (pangastritis) which predisposes them to gastric ulcers and gastric adenocarcinomas (Atherton, 2006).

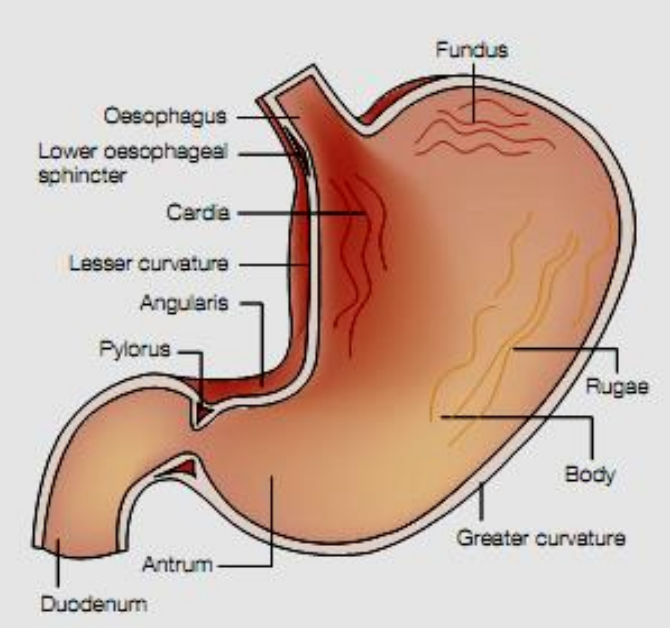


Figure 08: Gastric anatomy. Anatomical arrangement of the distal oesophagus, stomach and proximal duodenum. *Helicobacter pylori*-induced inflammation can occur at any site within the stomach. However, most intestinal-type and diffuse gastric adenocarcinomas associated with *H. pylori* occur in the gastric antrum, body, or (less likely) fundus. Oesophageal adenocarcinomas — which are a complication of gastroesophageal reflux disease and Barrett' s oesophagus, and are inversely related to the presence of *H. pylori*— occur in the distal oesophagus, just above and/or involving the lower oesophageal sphincter (Peek and Blaser, 2002).

2.6 *Treatment of H. pylori infection*

The spontaneous decline in the prevalence of *H. pylori* infection in developed countries to 10%–15% allows the remaining non-malignant gastroduodenal diseases associated with infection to be addressed with antimicrobial treatment.

2.6.1 *First-line therapy*

According to European and North American guidelines, there is a first-line therapy for treating *H.pylori* infection. It consists of a standard triple therapy including a Proton pump inhibitor (PPI) or ranitidine bismuth citrate, with any two antibiotics among amoxicillin, clarithromycin and metronidazole given for 7-14 days to adults. The PPI-based Triple therapy is also performed worldwide for *Helicobacter pylori*-associated diseases in childhood. A triple regimen with amoxicillin and metronidazole is acceptable where there is primary resistance to clarithromycin. In children with chronic *H. pylori* gastritis, eradication should be considered if they have gastric atrophy or a family history of gastric cancer. The duration of triple therapy vary in the American College of Gastroenterology guidelines (10-14 days) and in the Maastricht III Consensus Report guidelines (7 days) (Malfertheiner 2007; Chey 2007; Kato, 2009).

Even with the recommended triple regimens, *H. pylori* eradication failure is still seen in more than 20 % of patients. The failure rate for first-line therapy may be higher in actual clinical practice, owing to the indiscriminate use of antibiotics. In part this is due to increasing Clarithromycin resistance. In areas with a high prevalence of clarithromycin-resistant *H. pylori* Infection (>20%), clarithromycin should be substituted by metronidazole. In patients allergic to penicillins, the standard first-line triple-therapy includes a PPI, clarithromycin and metronidazole. A systematic review and meta-analysis of eight randomized trials (n= 1,679) compared the efficacy and tolerability of clarithromycin triple and bismuth quadruple therapies as first-Line treatment of *H. pylori*. Eradication was achieved in rates of 78.3 % vs. 77.0 % in patients allocated to quadruple and triple therapy, respectively. There was no statistically significant difference inside effects yielded by bismuth quadruple vs. Clarithromycin triple therapy. Patient compliance is similar for Quadruple and triple therapies (Luther 2010).

As an alternative initial treatment a sequential regimen was proposed a proton-pump inhibitor plus amoxicillin for 5 days followed by a PPI plus clarithromycin and an imidazole agent for more 5 days. The rationale for this more complicated approach is that amoxicillin may weaken bacterial cell walls in the initial phase of treatment, preventing the development of drug efflux channels that inhibit such drugs as clarithromycin from binding to ribosomes. This may help to improve the efficacy of clarithromycin in the second phase of treatment. This regimen was reported to achieve an eradication rate of 93 % versus a rate of 77 % with standard triple therapy, in a meta-analysis of 10 randomised trials involving 2747 patients in Italy (Jafri , 2008; Gatta , 2009).

2.6.2 *Second line therapy*

Treatment failure is often related to *H. pylori* resistance to clarithromycin or metronidazole (orbothagents). Then, the choice of second-line treatment depends on which treatment is used initially. The recommended standard second-line therapy is a quadruple regimen composed of tetracycline, metronidazole, a bismuth salt and a PPI. Quadruple regimens seem more effective as second-line treatment than triple regimens when a first-line triple regimen has failed to eradicate the infection. However, the evidence is limited in the comparison of second-line quadruple versus triple regimens, because most triple regimens did not contain a nitroimidazole. Besides, bismuth salts are not available in some countries, and tetracycline and metronidazole induce more frequently adverse effects and Interactions in comparison with other antibiotics. In the case of failure of second-line therapy, the patients should be evaluated using a case-by-case approach (Leontiadis , 2010).

2.6.3 Third and fourth line therapies

Patients in whom *H. pylori* infection persists after a second course of treatment and for whom eradication is considered appropriate should be referred to a specialist with access to facilities for culturing *H. pylori* and performing sensitivity testing and experience with alternative treatments for the infection. Even after two consecutive failures, several studies have demonstrated that *H. pylori* eradication can finally be achieved in almost all patients if several rescue therapies are consecutively given. European guidelines recommend a third-line treatment based on the microbial antibiotic sensitivity (Malfertheiner , 2007). *H. pylori* isolates after two eradication failures are often resistant to both metronidazole and clarithromycin. Several regimens have been reported to be effective eas salvage therapy in case series. The alternative candidates for third-line therapy are quinolones (levofloxacin, moxifloxacin), tetracycline, rifabutin and furazolidone. High-dose PPI plus amoxicillin and levofloxacin or rifabutin therapy has been associated with high rates of eradication. However, caution is warranted in the use of rifabutin, which may lead to resistance of mycobacteria in patients with preexisting mycobacterial infection. Besides, rifabutin has been associated with rare but potentially serious myelotoxicity and ocular toxicity (Chey , 2007). Due to these disadvantages, rifabutin-based rescue therapy constitutes an empirical fourth-Line strategy only after multiple previous eradication failures with key antibiotics such as amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin (Gisbert, 2008).

However, treatment of infection is challenged by, for example, the rapid rate with which the bacteria acquire resistance to the drugs, poor compliance, an excessively high bacteria load, impaired mucosal immunity, early re-infection and the presence of intracellular bacteria (Mégraud and Lamouliatte, 2003). Thus, there is still a need for new compounds and therapeutic regimens to eradicate *H. pylori* infection.

The table 05 shows the different options of therapy and the most common regimens used respectively (Wannmacher, 2011).

2.7 *Biocontrol of H.pylori infection by probiotics*

Despite high efficacy of antibiotic treatment regimens applied for the eradication of *H. pylori*, some limits have been reported such as high cost, side effects and antibiotic resistance. For this reason triple therapy is not recommended in most infected subjects such as "healthy" asymptomatic carriers and dyspeptic patients without ulcers (Dooley 1989; Malfertheiner 2002; Kashiwagi, 2003; Plummer 2004).

Defined previously, probiotics are live micro-organisms that when ingested in adequate amounts exert health benefits on the host, and they have been used in the management of various medical disorders and particularly gastrointestinal pathologies. At present the most studied probiotics are lactic acid-producing bacteria particularly *Lactobacillus* species. The most well documented effects of probiotics on gut diseases coming from clinical trials as well as from experimental studies concern acute infectious diarrhoea or pouchitis.

However, their use has been proposed and is still investigated against *H. pylori* infection (Rolfe, 2000; Gill and Guarner, 2004).

The clinical outcome of *H. pylori* infection is determined by several factors including the type of *H. pylori* strain, the extent of inflammation and the density of *H. pylori* colonization. Furthermore, the risk of peptic ulcer disease development and gastric cancer increases according to increasing level of infection. Therefore, permanent or long-term suppression of *H. pylori* could decrease the risk of *H. pylori*-related diseases development. Consequently, there is increasing interest in developing low-cost large-scale alternative solutions to prevent or decrease *H. pylori* colonization. In this respect probiotics may close the therapeutic gap (Ernst and Gold, 2000; Yamaoka , 1999).

H. pylori strains are known to produce urease, which can hydrolyse urea to ammonium species, resulting in elevated pH in the stomach and promoting adhesion of microorganisms (Sullivan and Nord 2002). Thirabunyanon , (2009) found that the potential probiotics, *Enterococcus faecium* RM11 and *Lactobacillus fermentum* RM28, isolated from fermented dairy products could inhibit the growth of *H. pylori*. In an investigation (Turnbaugh , 2006), patients infected with *H. pylori* received milk containing the probiotic *Lactobacillus casei* Shirota strain continually for 6 weeks. The results showed that urease activity declined in 64% of the patients who consumed the fermented milk, as compared with 33% for the control group. Similar results were obtained by Myllyluoma (2007), who concluded that decreasing urease and gastrin-17 activities were found in *H. pylori*-infected patients who consumed a probiotic combination of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* JS and *Bifidobacterium lactis* Bb12 for 8 weeks.

Table 05: Options and regimens used for the eradication of *H. pylori* infection (Wannmacher, 2011).

Options	
Standard First-line therapy	
Triple therapy (70-86%):	PPI, amoxicillin, clarithromycin (77%) or PPI, amoxicillin, metronidazole (In infection resistant to clarithromycin) or PPI, clarithromycin, metronidazole (97%) (In patients allergic to penicillin)
Alternative First-line therapy	
Sequential therapy:	Days 1–5: PPI and amoxicillin Days 6–10: PPI, clarithromycin, metronidazole (93%)
Triple therapy:	PPI, levofloxacin, amoxicillin (74.2 %) (In dual resistance to clarithromycin and metronidazole)
Quadruple therapy:	PPI, bismuth, metronidazole, tetracycline (78.3%) (In areas where the resistance to clarithromycin or metronidazole is >20% or in patients with recent or repeated exposure to clarithromycin or metronidazole)
Standard Second-line therapy	
Quadruple therapy:	PPI, doxycycline, metronidazole, bismuth salt (76%) or PPI, doxycycline, metronidazole, amoxicillin (When bismuth salt is not available)
Alternative Second-line therapy	
Triple therapy:	PPI, metronidazole, amoxicillin (75%) or PPI, metronidazole, doxycycline (75%) (In patients with penicillin allergy) or PPI, levofloxacin, amoxicillin (76.9%) or PPI, furazolidone, amoxicillin (76.1%)
Third-line treatment	
	High-dose PPI, amoxicillin, tetracycline, levofloxacin, furazolidone-containing regimen (65.5%) or rifabutin
Regimens	
Standard initial treatment (use one of the following three options)	
Triple therapy (7–14 days)	PPI - healing dose[a], twice/day + Amoxicillin - 1 g, twice/day [b] + Clarithromycin - 250-500 mg, twice/day
Sequential therapy (10 days)	Days 1-5: PPI - healing dose a, twice/day + Amoxicillin - 1 g, twice/day Days 6-10: PPI - healing dose a, twice/ day + Clarithromycin - 500 mg, twice/day + Tinidazole - 500 mg, twice/day
Quadruple therapy (10–14 days)	PPI - healing dose a, twice/day + Bismuth salt - 120 mg, four times/day + Tetracycline - 500 mg, four times/day + Metronidazole - 250 mg, four times/day
Second-line therapy (use one or the other)	
Triple therapy (7–14 days)	PPI - healing dose a, once/day + Amoxicillin -1 g, twice/day + Metronidazole – 400-500 mg, twice/day
Quadruple therapy	as recommended for initial therapy

[a] Omeprazole: 20 mg; esomeprazole: 20 mg-40 mg; rabeprazole: 20 mg; pantoprazole: 40 mg; lansoprazole: 30 mg ; tenatoprazole: 40 mg.

[b] If the patient has penicillin allergy, use metronidazole (400-500 mg, twice/day) and use clarithromycin at reduced dose of 250 mg twice per day.

The suppression of *H. pylori* binding to the glycolipid receptors by the probiotic *Lactobacillus reuteri* has been reported (Mukai 2002). Lin (2009) proposed that lactic acid bacteria isolated from commercial food products can inhibit *H. pylori* infection at the adhesion sites of human gastric epithelial AGS cells. Sgouras (2004) found that *Lactobacillus casei* Shirota was highly effective in reducing *H. pylori* colonisation in the antrum and body mucosa in a mouse model while *Lactobacillus gasseri* OLL2716 was shown to be effective against *H. pylori* infection in children (Shimizu 2002).

Similarly, Wang (2004) indicated that regular consumption of yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 may be effective in inhibiting *H. pylori* infection in humans. The outcome of using two combined probiotic strains of *Bacillus subtilis* and *Enterococcus faecium* for *H. pylori* eradication in patients were observed. These actions of the probiotic group were found to have a higher eradication rate (83.5%) than that of the control group (73.3%) (Park 2007).

2.8 *Probiotic molecules that Inhibit H. pylori*

Two main types of substances have been implicated in the inhibition of *H. pylori* by lactic acid bacteria: short chain fatty acids (SCFAs) and bacteriocins. SCFAs such as formic, acetic, propionic, butyric and lactic acids are produced during the metabolism of carbohydrates by probiotics and have an important role in decreasing pH. Bhatia (1989) were the first to observe an antagonistic effect of a *Lactobacillus* strain against *H. pylori* and to implicate SCFAs in this effect. A dose-dependent inhibition of *H. pylori* growth has been observed with acetic and lactic acid, the later demonstrating the most intense effect (Midolo 1995). The amounts of lactic acid released by strains of *Lactobacillus, Bifidobacterium* and *Pediococcus* (50–156 mM) correlated with the intensity of their inhibitory effect against *H. pylori*. Such antimicrobial activity could be due not only to a direct effect on *Helicobacter* but also to the inhibition of its urease activity, as shown with the high lactic acid producers *L. salivarius* and *L. casei* Shirota (Aiba 1998; Sgouras 2004).

Interestingly these strains, when administered to colonized mice, induced a significant decrease in the counts of *H. pylori* in the body and antral mucosa compared with untreated animals, and resulted in a concomitant reduction in the associated gastric inflammation. A traditional yogurt originating from Asia was evaluated for its bactericidal activity against *H. pylori* (Oh 2002) both the yeasts (*Kluyveromyces lactis* and *Issatchenkia orientalis*) and lactobacilli (*L. crispatus and L. kefiri*) present in the product exert independently an anti-*H. pylori* activity. This yogurt had high levels of lactic acid and formic acid but only the latter was found to have a consistent inhibitory effect against *H. pylori*.

Bacteriocins are compounds with potential anti-*H. pylori* activity. They are small, heat-resistant peptidic structures with antimicrobial activities, which are synthesized by several bacterial species including lactic acid bacteria. Many bacteriocins have

been characterized and some of them, such as nisin, are added to foodstuffs to decrease the risk of contamination by pathogens. The release of bacteriocins with anti-*H. pylori* activity has been chiefly studied in *Lactobacillus*, but probiotic strains of *Enterococcus faecium* (Tsai 2004), *Bacillus subtilis* (Pinchuk 2001) and *Bifidobacterium* (Collado 2005) could also produce heat-stable proteinaceous compounds capable of inhibiting the growth of both antibiotic-resistant and sensitive strains of *H. pylori*. Kim (2003) evaluated, by microdilution assay, the anti-*H. pylori* activity of seven bacteriocins produced by lactic acid bacteria, including nisin A, pediocin PO2, leucocin K and various types of lacticins. Lacticins A164 and BH5 produced by strains of *L. lactis* had the most potent activity, with minimum inhibitory concentrations varying from 0.097–0.390 mg/L to 12.5–25 mg/L depending on the strains of *H. pylori* tested, indicating a strain-dependent sensitivity of this pathogen. In a co-culture experiment, Lorca (2001) showed that after 24 h of culture an autolysate of *L. acidophilus* CRL 639 induced the release of a proteinaceous compound and the subsequent mortality of all *H. pylori* after 48 h.

The supernatant of a culture of the probiotic strain, *L. johnsonii* La1 was shown to inhibit both the urease activity and growth of *H. pylori*. This inhibitory activity remained functional even when *H. pylori* was bound to HT-epithelial cells; furthermore, this activity was not observed with La10, another strain of *L. johnsonii*, indicating that it is strain-specific, and was independent of the presence of the cag pathogenicty island in *H. pylori* strains (Michetti 1999). The supernatant inhibitory activity was heat resistant, dialysable and not affected by 10 mM urea, being therefore compatible with the presence of a bacteriocin. Although the molecule responsible for this effect was not purified and characterized by the authors, it could be lactacin F, a two-component class II bacteriocin produced by *L. johnsonii* and composed of LafA and LafX peptides which may combine to form a pore in the membrane of sensitive bacteria, resulting in the efflux of intracellular ions and the eventual death of the pathogen in this case (Dalmau 2002). Other findings were observed by Coconnier (1998) incubating *H. pylori* with the culture supernatant of *L. acidophilus* LB; the viability of the pathogen as well as its urease activity and its binding to the HT29-MTX cell line decreased in a dose-dependent manner. This effect was independent of the presence of lactic acid and the pH and was not suppressed by heating at 100 °C for 1 h, supporting the presence of at least one bacteriocin. Interestingly, *H. pylori* was morphologically affected by the incubation with supernatant of the LB culture, changing from its characteristic curved form with polar flagella to U-shaped and decreased size, considered as pre-coccoid. No effects on *H. pylori* were observed by these authors using the culture supernatant of another well-known probiotic strain, *L. rhamnosus* GG (LGG) (Coconnier 1998).

Clinical trials based on the resistance of certain lactobacilli to the low gastric pH, suggesting possible adhesion and transient residence in the human stomach, clinical trials have been undertaken in order to determine the effect of probiotic treatments on *H. pylori* infection. Several trials investigated this effect either using probiotic

formulations alone or in combination with antibiotics were published. The most frequently used strain was *L johnsonii* La1, either in a fermented milk formula or as a free cell culture supernatant. Other probiotics used were *L. casei*, *L. brevis* and *L. gasseri*. Clinical trials investigating the effects of probiotic action on *H. pylori* infection were summarized in Tables 06 and 07.

Table 06: Some clinical trials using probiotics in association with antibiotics in the treatment of *H. pylori* colonization (Gotteland , 2006).

Study design therapy	**Subjects (n)**	**Eradication**	**Probiotic**	**Results**
R, O	Dyspeptic adults (120)	Rabeprazole, clarithromycin, amoxicillin	*L. acidophilus* LB for 10 days	E.R.: ↑ A.E.: no effect
R, O	Asymptomatic adults (120)	Pantoprazole, clarithromycin, tinidazole	*L. rhamnosus* GG, 1.2 10^{10}/day for10 days	E.R.: no effect A:E.: ↓
R, O	Dyspeptic subjects (160)	Lansoprazole, clarithromycin, amoxicillin	*L. acidophilus* LA5 + *B. lactis* Bb12, 10^{10} /day for 4 weeks	E.R.: ↑
DB, P, R	Asymptomatic adults (85)	Rabeprazole, clarithromycin, tinidazole	*L. rhamnosus* GG *S. boulardii Lactobacillus* LA5 + *B. lactis* Bb12, for 2 weeks	E.R.: no effect A:E.: ↓
DB, R, P	Asymptomatic adults (52)	clarithromycin	*L. johnsonii* LA1 acidified milk, 180 mL/day for 3 weeks	E.R.: no effect Lower gastric density of Hp Lower gastric inflammation
R	Dyspeptic patients with resistant *H. pylori* infection (70)	Esomeprazole or pantoprazole, Ranitidine bismuth citrate, amoxicillin and tinidazole	*L. casei* DG, 1.6 · 1010/day for 10 days	E.R.: no effect A:E.: ↓

DB, double-blind; R, randomized; P, placebo-controlled; O, open; E.R., eradication rate; A.E., adverse effects.

Table 07: Some clinical trials using probiotics alone in *H. pylori*-colonized subjects (Gotteland , 2006).

Study design therapy	Subjects (n)	Probiotic	Results
R, DB, P	Asymptomatic adults (20)	*L. johnsonii* La1 culture supernatant + omeprazole	^{13}C-UBT results ↓
R, DB, P	Asymptomatic children (236)	*L. johnsonii* La1 *L. paracasei* ST11 1.8 · 109/day for 4 weeks	^{13}C-UBT results ↓
	Asymptomatic adults (12)	*L. johnsonii* La1 8 · 108 every 2 h during 14 h for 2 weeks	^{13}C-UBT results ↓
R, DB, P	Asymptomatic adults (50)	*L. johnsonii* La1 acidified milk for 3 and 16 weeks	↓ gastritis ↓ gastric density of *H. pylori*
P	Dyspeptic adults (70)	AB-yogurt with *L. acidophilus* LA5 + B. lactis Bb12, 10^{10}/day for 4 weeks	^{13}C-UBT results ↓
R	Asymptomatic children (254)	*S. boulardii* (500 mg/day) + inulin (10 g/day) *L. acidophilus* LB (heat inactivated), 10^{10}/day for 8 weeks	Eradication of 12% with Sb, 6.5% with LB
O	Asymptomatic adults (27)	Yogurt with *L. casei* 03, *L. acidophilus* 2412, *L. acidophilus* ACD1 + starter strains, 175 mL/day for 30 days	No effect
R, P	Asymptomatic adults (14)	*L. casei* Shirota, 1.95 · 10^{10}, for 3 weeks	No clear effect
P	Asymptomatic adults (31)	*L. gasseri* OLL 2716, 2.2 · 10^{9}/day, for 8 weeks	^{13}C-UBT results: ↓ Pepsinogen1/2 : ↓
O	Symptomatic children (12)	*L. gasseri* OLL 2716 for 8 weeks	^{13}C-UBT results: no effect H. pylori stool antigen: ↓ Pepsinogen1/2: ↓

DB, double-blind; R, randomized; P, placebo-controlled; O, open; E.R., eradication rate; A.E., adverse effects.

II.

Experimental Section

Chapters

03 & 04

Pollen

Microbiology

3. Microbiological Sanitary Aspects of Pollen

3.1 INTRODUCTION

Stamens are the male reproductive organs of flowering plants. They consist of an anther, the site of pollen development, and in most species a stalk-like filament, which transmits water and nutrients to the anther and positions it to aid pollen dispersal. Within the anther, male sporogenous cells differentiate and undergo meiosis to produce microspores, which give rise to pollen grains, whereas other cell types contribute to pollen maturation, protection, or dispersion Scott *et al.*, 2004). Faegri and Van der Pijl (1966), defined two major types of pollen dispersal: *Biotic pollination* in which the pollen dispersal agent is an animal (i.e., either an invertebrate or a vertebrate); and *abiotic pollination* where pollen is dispersed by an inanimate physical agent, such as wind or water. Mutually beneficial ecological relationships have been established between bees and plants. There are about 250 thousand species of flowering plants on earth, many of which have amazingly complex relationships to bees and other pollinators including flies, beetles, moths, butterflies, birds and bats (Shrestha, 2008). Honeybees, mainly *Apis mellifera*, remain the most economically valuable pollinators of crop monocultures worldwide and yields of some fruit, seed and nut crops decrease by more than 90% without these pollinators (Klein *et al*., 2007). Honeybees remove pollen from an anther by using their tongue and mandibles, mixed it with salivary secretions from her mouth, and transfer it to the corbicula, or "pollen basket", on her posterior pair of legs. To prevent bacterial growth and delay pollen germination various enzymes produced by worker bees are added to the pollen as it is packed into broad-free combs (Stanciu *et al*., 2009). Historically, bee-collected pollen has been used as a food and medicine by various civilizations, as different nutritional and medicinal traits were attributed to it (Crane, 1999). Nowadays, tons of pollen, either processed or unprocessed, are sold for human and animal consumption. Pollen is a riche plant product, containing carbohydrates, proteins, enzymes, fatty acids, minerals, and vitamins. However, unlike honey, from the anther dehiscence to comb cells, pollen is exposed for microbial contamination. This contamination can be attributed to various factors and sources, honey bees, weather, plant materials, insects and animals, humans and their agricultural devices. Numerous studies were revealed the occurrence of fungal and bacterial species in pollen, but little is known about its microbiological quality. For this reason, pollen quality control is of utmost importance, particularly if the pollen is intended for human consumption. The aim of this preliminary investigation is to examine microbial quality of tow pollen collected by honeybees and one hand collected pollen from Algeria.

3.2 MATERIALS AND METHODS

3.2.1 *Sampling and storage*

Pollen samples were collected from different regions in Algeria during flowering season (from March to May 2010). Pollen sample 1 (PS1) was collected from Sétif (Midle east of Algeria), pollen sample 2 (PS2) was from Biskra (North Algerian Sahara), whereas pollen sample 3 (PS3) was from Blida (North of Algeria) (Table 8). Samples were obtained from clean ecological regions situated in agricultural lands. PS1 and PS 3 were trapped from honey bees workers, whereas, PS2 was collected from date palm trees by hands. All the samples were transported to the laboratory in sterile glass vials and conserved at 4°C until use.

Table 08: Topographic data of the nearest weather stations for sampling sites.

Sampling site	Weather station	Latitude	Longitude	Altitude
Blida	603900 (DAAG)	36.68	3.25	25 m
Biskra	605250 (DAUB)	34.8	5.73	87 m
Sétif	604450 (DAAS)	36.18	5.41	1038 m

3.2.2 *Microbial and weather parameters analysis*

Twenty-five grams of each sample of pollen were diluted in 225 mL of peptone saline solution (1 g/L peptone and 8.5 g/L NaCl) and homogenized for 10 min by chaking in an orbitrary chaker (200 rpm). After 30 min at room temperature, serial dilutions of suspension were made in a saline solution (8.5 g/L NaCl) and analyzed. The formed colonies on the plates were counted and expressed as Log colony forming units/g (Log CFU/g). Another 25 g were diluted in 225 mL of buffered peptone water for the isolation of *Salmonella* spp.

Total aerobic microflora: It was determined by spreading 0.1 mL from the appropriate dilution onto sterile disposable Petri dishes to which Plate count Agar (Merck, 5463) was poured. Plates were incubated aerobically at 30° C and counted after 3 days.

Total anaerobic microflora: The plat count agar was used to enumerate anaerobic microflora by spreading 100 µL from the appropriate dilution on plate count agar. The plates were incubated at 30°C for 3 days under anaerobic conditions using candle jars.

Total mold and yeasts: From appropriate dilutions. 100 µL were surface plating onto sterile disposable Petri dishes to which was added Potatoes Dextrose agar (SIGMA, P2181). Plates were incubated at 28°C and counted after 7 days.

Total coliforms: a 100 µL aliquot of the appropriate dilution were transferred into Petri plates and poured with Violet Red Bile Glucose agar (VRBG Agar). Solidified plates vere overlaid with 5 mL of VRBG agar. Plates were incubated at 35°C and typical colonies (dark red, 0.5 mm. or more in diameter) were counted after 24 h.

Staphylococcus aureus was determined by spreading of 100 µL from the appropriate dilutions on Baird-Parker agar (Merck, 5406) and incubated at 37°C for 48 h.

***Salmonella* spp.:** Pre-enrichment was conducted from samples diluted in 225 mL buffered peptone water incubated at 37°C for 18 h. Secondary selective enrichment was performed in Rappaport-Vassiliadis peptone broth (41°C for 24 h) and Muller-Kaufmann tetrathionate broth (37°C for 24 h), and plating on Brilliant Green Agar and XLD agar incubated at 37°C for 24 h.

Weather parameters (temperature, relative humidity and precipitation amounts) as well as topographic indicators (Latitude, Longitude, and Altitude) data were obtained from three weather stations [Blida, 603900 (DAAG); Biskra, 605250, (DAUB); and sétif, 604450 (DAAS)] (Tables 7 and 9).

3.3 RESULTS

Microbial content of three pollen samples is presented in Table 9 and Figure 9. It should be emphasized that microbial enumeration varies considerably. Statistical analysis of the data showed that total aerobic counts were significantly higher in pollens from Sétif and Blida (620 x 10^3 and 470 x 10^3 CFU/g respectively), significantly lower in pollen from Biskra (10^3 CFU/g).

Table 09: Microbial enumeration of three pollen samples collected in Algeria from March to May 2010. (Results were expressed as CFU/g or Log_{10} CFU/g of wet pollen).

	Origin of Pollen Samples					
	PSa (Sétif)		PSb (Biskra)		PSc (Blida)	
	10^3 CFU/g	Log_{10} CFU/g	10^3 CFU/g	Log_{10} CFU/g	10^3 CFU/g	Log_{10} CFU/g
Total aerobic counts	620	5,80	01	3,00	470	5,67
Total anaerobic counts	600	5,77	ND	ND	82	4,91
Moulds and Yeasts	400	5,60	50	4,69	230	5,36
Staphylococcus aureus	00	ND	00	ND	27.3	4,43
Salmenella spp.	00	ND	00	ND	00	ND
Total coliforms	300	5,47	01	3,00	19	4,27

Similarly, total mould counts were 400 x 10^3/g for pollen from Sétif, 50 x 10^3/g for pollen from Biskra and more than 200 x 10^3/g for pollen from Blida. *Salmonella* was not detected in all samples, whereas, staphylococci were recoded only in pollen from Blida (27.3 x 10^3/g). Like total aerobic counts and mould counts, higher levels of coliforms were observed in pollen from Sétif (300 x 10^3/g), 19 x 10^3/g and 10^3/g for Blida and Biskra respectively. Statistical analysis shows that enumeration of the different microbial groups within each sample has extremely significant differences ($P < 0.0001$), either for normal set or transformed data. Also, from region to region or from sample to another, microbial content differs greatly (P = 0, 0037; < 0.05). Considering now the weather conditions, significant differences in relative humidity and precipitation amount (P <0.05) were recorded between the regions sétif/Biskra and Blida/Biskra, whereas, there was no statistical differences between weathers of Sétif and Blida. In addition, temperature does not differs between all area sampling (P >0.05). Analysis of correlation between the organisms enumerated showed that total aerobic plat counts correlated (r= 0.34) only with total coliforms and relative humidity (RH) (r= 0.12) in pollen from Sétif and correlated (r= 0.31) with total coliforms and (r= 0.69 and r= 0.69) with RH and precipitation amount respectively in pollen from Biskra. In Blida's pollen total aerobic plate counts correlated (r= 0.25) with staphylococci and both (r= 0.74 and r= 0.47) with RH and precipitation amount. In addition, total molds and yeast counts correlated (r= 0.58) with temperature in pollen from Biskra, and in pollen from Blida correlated (r= 0.37) with relative humidity.

Table 10: Recorded data for temperature (°C), relative humidity (%), and precipitation amount (mm) at three measurement weather stations.

	Temperature (°C)			Humidity (%)			Precipitation amount (mm)		
	Sétif	Biskra	Blida	Sétif	Biskra	Blida	Sétif	Biskra	Blida
Jan.	6.1	12.6	11.7	79.2	55.7	73.8	32.26	15.75	45.73
Feb	7.4	14.6	13.4	79.3	52.1	67.8	43.94	17.78	48.53
March	9.4	18.3	14	73.1	44.4	71.5	39.62	23.87	101.35
Aprile	12.5	22.1	16.1	74.2	46.3	78.6	55.12	30.23	32.76
May	13.6	24.4	17.8	69.7	33.9	67.2	73.66	7.11	23.36
Mean ±	9,8±	18,4±	14,6±	75,1±1,8	46,48±	71,78±	48,92±	18,94±	50,34±
SEM	1,43	2,21	1,065	4	3,73	2,09	7,2	3,89	13,53

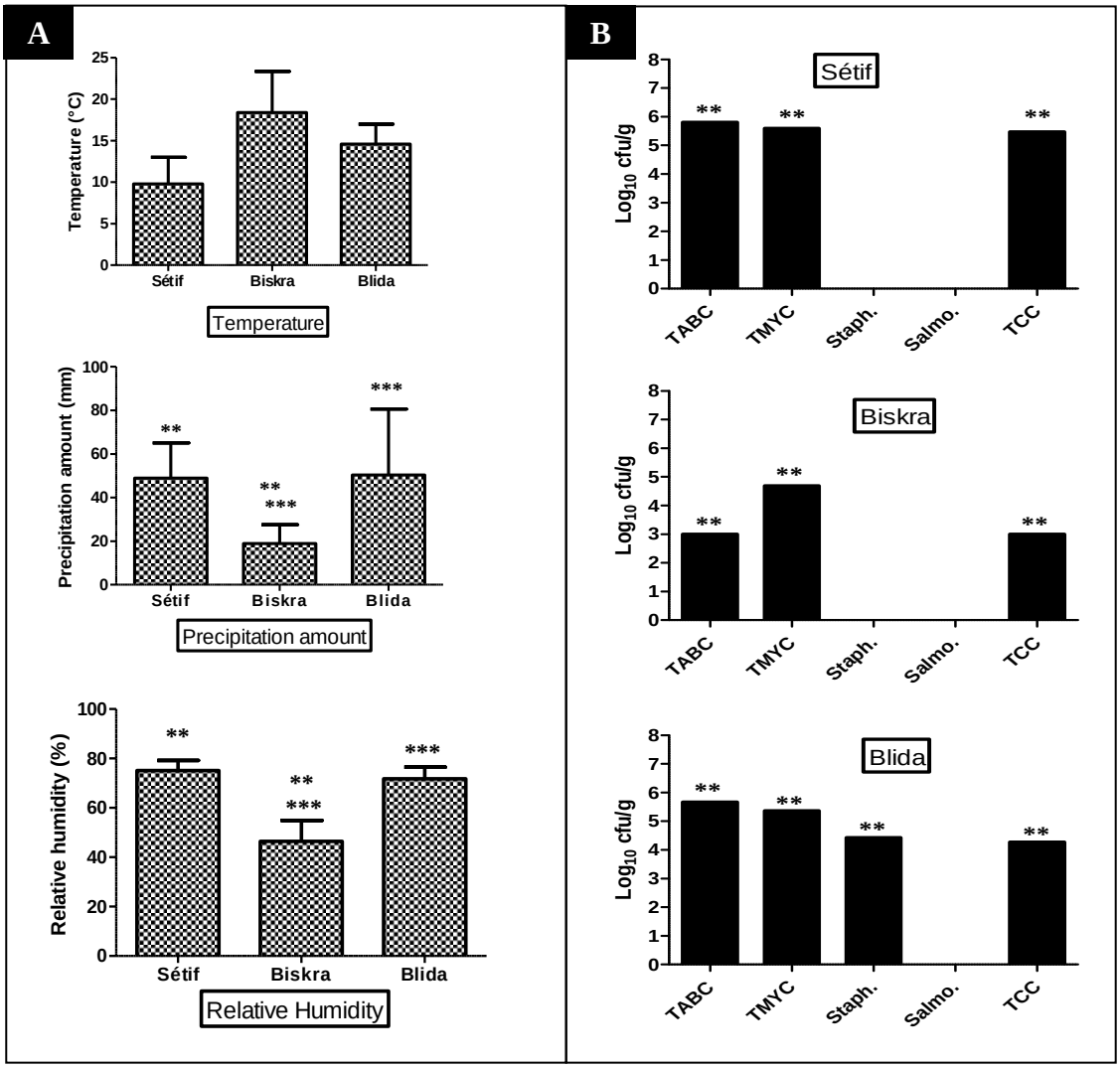


Figure 09: A; recorded temperature (°C), precipitation amount (mm) and relative humidity (%), at the sampling sites (sétif, Biskra, and Blida), columns represent means of five values, upper bares indicate SD (standard deviation), (***) and (**) indicates that *P* was <0.01 between represented data. B; represented Log_{10} CFU/g of different microbial groups in pollen samples. (TAPC, total aerobic plat count; TMYC, total molds and yeast count; Staph., *Staphylococcus aureus;* Salmo., *Salmonella* spp.; TCC, total coliforms counts), and (**) indicates that *P* was <0.05.

3.4 DISCUSSION

Microbial contamination of herbs and/or products may results from improper handling during production, collection and packaging (Kosalec *et al*., 2009). World Health Organization (WHO) (2007) contaminant guidelines propose that contamination should be avoided and controlled through quality assurance measures such as good agricultural and collection practices (GACP) for medicinal plants, and good manufacturing practices (GMP) for herbal medicines. In recent years, only a small percentage of medicinal plants are collected from the wild, and there are too few data to compare biological contamination between wild and cultivated medicinal herbs. Guidelines, such as the GACP and GMP, aim at reducing the overall risk of contamination, not only biological (Kosalec *et al*., 2009). According to Campos *et al*. (2008), pollens should have the following microbial aspects: absence of *Salmonella*/10g; absence of *Staphylococcus* and *Escherichia coli* /01 g; TAPC could not exceed than 10^5 CFU /g; TMYC should be less than 5. 10^4 CFU/g; and the maximum of enterobacteria is 100 CFU/g. Comparing, however, these recommendations with our results reveals clearly that bee-collected pollens (Sétif and Blida) have a poor microbial quality, in contrast to hand collected pollen (Biskra) which have an acceptable criteria. In Algeria, peoples consume pollens. Great populations purchase it from beekeepers and use it as dietary supplement, mainly with dairy products such as milk. However, numerous studies have been conducted to determine microorganisms associated with pollen. Gilliam (1979a), identified more than six yeast genera (*Cryptococcus, Kloeckera, Candida, Rhodotorula, Torulopsis, Hansenela*) from almond pollen. Later, *Bacillus subtilis, B. megaterium, B. licheniformis,* and *B. circulans* were isolated from the same pollen of almonds (Gilliam 1979b). In addition, the majority of molds identified from pollen by Gilliam *et al*. (1989, 1990) were penicillia, mucorals and aspergilli. Pollen is sterile inside anther, thus its microbial contamination can be attributed to plant materials, environment, insects (eg, Honeybees), and humans and their agricultural devices. Naturally, pollen grains contain antimicrobial substances (flavonoids, phenolic acids, and other phytochemicals) (Carpes et al., 2005; Basim *et al*., 2006) as well as microbial spore germination inhibitors (Pandy *et al*., 1983). As reviewed by Gonzâlez *et al*. (2005), growth and sporulation of fungi, both on standing crops and in stored grains, are largely dependent on environmental factors. The most important determinants are probably a_w and temperature. On this fact, results of this work point out that differences of microbial content between and within pollen samples were related to relative humidity and precipitation amount, as temperature does not differs greatly between regions. World wide, pollen processing includes harvesting, drying, cleaning, packaging and storage (Murray, 2000). Gonzalez *et al*. (2005) concluded in their work that the most critical stage is pollen collection from traps. The longer period of collection the highly contaminated pollen was obtained. In contrast, the fact that corbicular pollen is a mixture of pollen, nectar and salivary secretions of

honeybees (Keller *et al.*, 2005). Furthermore, Gilliam *et al*. (1983) reported the occurrence of Gram-negative and Gram-positive bacilli as well as Gram-positive cocci in nectar. In addition, fungi are found in association with honey bee colonies where they persist on nectar, pollen, in colony debris, and inside bees themselves (Benoit *et al.*, 2004). And from the results of this work, it seems that honeybees are the main source and/or carrier for pollen contamination. The high incidence of fungi in pollen grains studied in this work point out that raw pollen is not ready-to-eat directly without processing (drying). In Algeria, pollen is dried naturally by exposing it to sunlight or oven heated at a low temperature (less than 45°C). Serra and Alegret (1986) recommend the avoidance of natural pollen drying, because at low temperatures, fungal growth and mycotoxin production might occur. If raw pollen was stored without processing, fungi can flourish under appropriate conditions (humidity and temperature) as pollen is a suitable plant product either for fungal growth or mycotoxin production. However, mycotoxins production, such as ochratoxin A (OTA) and aflatoxins B_1, B_2, G_1, and G_2 by pollen associated fungi is known (Gonzalez *et al*., 2005). Aflatoxins and ochratoxin A are thermostable and carcinogenic molecules (Boudra and Bars, 1995; Pitta and Markaki, 2010), thus, pollen dried naturally or not may present a threat for human and animal health. Considering now bacterial content, however, the incidence of staphylococci in pollen from Blida maks it indesirable dietary supplement. *Staphylococcus aureus* remains a versatile and dangerous pathogen in humans. The microoorganism produce numerous toxins that are responsible for diverse syndroms and life-threatening diseases, as the staphylococci are well known for their multiresistance to antimicrobial drugs (Lowy, 1998). It is truly that analyzed pollen samples in this work contain high levels of enterobacteria and exceed recommended values. *Enterobacteriaceae* are a group of bacteria that can be found in many environments. They can be found in the intestinal tract of humans and animals. They can also be found in soil, vegetable matter and marine environments. The group includes both pathogenic and non-pathogenic bacteria. As they can be found in raw foods, their detection may not be an indication of fecal contamination and is inappropriate to test ready-to-eat foods containing raw components (particularly fresh fruits and vegetables) (NSWFA, 2009). But taking in mind the fact that enterobacterial cell membrane contains an antigenic, pyrogenic and thermostable molecule, the endotoxin or lipopolysaccharide (LPS). However, the bacterial endotoxin is released during bacterial multiplication or death and triggers a series of important biological events that lead to an inflammatory response and bone resorption (Silva *et al.*, 2007). Indeed, either raw or dried pollen may be considered as a potentially hazardous medicinal plant product, as it is exposed for contamination by toxigenic fungi and pathogenic bacteria such as aspergilli, penicillia and staphylococci. This contaminated pollen present a suitable environment and/or a suitable delivery system for mycotoxins and/or bacterial endotoxins. Peoples, especially in developing countries, continue to use pollen without the existence of international

microbiological quality guidelines and reglementations for pollen, as well as the ineffectiveness of pollen sanitization methods designed for human consumption, more studies are needed to understand the microbial content of pollen and to establish international microbiological quality parameters and standard processing protocols.

4. Microbiological Quality Control of Marketed Pollen

4.1 INTRODUCTION

For centuries, plant products have been used for a variety of functions. Trading of plants and plant products used for medicinal purposes were expanded in various societies. This situation probably stimulated by the fact that people believe that the consumption of natural products is healthier and safe than conventional medicine. As a plant product, pollen collected either by man or by honey bees, is used for nutritional and medicinal purposes. Because of its richness by proteins, amino-acids, carbohydrates, minerals, as well as vitamins, it was used as a dietary and a fortifying food. During the last few years, numerous pollen formulas are developed and marketed worldwide. Furthermore, researches that revealed the promising biological activities (therapeutic effects) of pollen extracts in recent years enhanced greatly the importance of pollen consumption in the society. In Algeria, as well as in other countries, pollen is used by peoples for various reasons, principally, as a weight raising food, general health wellbeing, as a fortifying agent and prostate hypertrophy treatment. In practice, apiarists collect pollens using pollen traps. These latter are non sterile and may contain various and different microbial populations originated during either manufacturing, transport, storage or handling. In addition, from flowers to beehives, pollen is exposed to microbial contamination by dust, air, insects, animals, and man. Most bee combs in Algeria, are not far away from industrial zones and metropolitan activities. Therefore, during their foraging, bees collect other man manufactured products, such as patisseries, sweet cakes; which may be contaminated by human or animal wastes. From another aspect, apiarists feed, usually, their bees, a home made syrup basically prepared from sugar, milk and water. These latter ingredients are sources for microbial contamination and suitable growth medium for bacteria and fungi. Bee-Pollen, after collection, will be dried, purified, packed, stored and marketed to the consumers without an effective hygienic and sanitary control. During each step of this important manufacturing chain, pollen continues to load and kept the original microbial flora as the first step, drying (40 °C max), is insufficient to removing and/or reducing microbial populations. Pollen is a suitable carrier for Gram positive and Gram negative bacteria, fungi and yeasts. Fungi and some bacterial species are spore formers. Also, growth and aflatoxin production, carcinogenic secondary mycotoxins, have been reported by Pitta and Markaki (2010). In addition, our preliminary study (Belhadj *et al.*, 2012) on pollen microbial flora revealed the existence of elevated numbers of molds, coliforms, *Staphylococcus aureus* as well as aerobic and anaerobic microorganisms. Bacterial spores are resistant to thermal treatment. Some of these bacteria like *Bacillus cereus* and *Clostridium perfringens* are recognized as potential pathogenic organisms and have been incriminated in food poisoning (Kunene *et al*., 1999; Miwa *et al.*, 1999). Pollen formation (flowering) and harvesting occur during warmth and humid seasons. Such environmental conditions favour microbial contamination and proliferation. Pollen can be classed as important vehicle for various micro-organisms implicating possible health problems for consumers and shelf life problems. Foodborne diseases are

perhaps the most wide-spread health problem in the contemporary world and an important cause of reduced economic productivity (WHO, 2005). In most of the cases of foodborne illness, the pathogenic effect occurs in the alimentary tract giving rise to symptoms of diarrhoea and vomiting. Since it is a natural product, all pollen constituent can be degraded by bacteria and fungi. Unscientific methods of harvesting, inappropriate drying and purification, unsuitable packing, storage and transportation, inadequate hygiene of producers and congenital climatic conditions render the raw material prone to infestations and exposed it to many microbial contaminants. Raw plant materials are most often degraded by microorganisms before harvesting, during handling and after prolonged storage (Mathe, 1995; Kenneth, 1989). The presence of sufficient numbers of microorganisms can be harmful to consumers. As a result of fungal contamination, the risk of mycotoxin production, especially aflatoxins, should be taken into consideration in the manufacturing process because of the proven mutagenic, carcinogenic, teratogenic, neurotoxic, nephrotoxic, immunosuppressive activities (Refai, 1988; Scimeca, 1995; FAO, 2000; Hoaler, 2000). From a legal point of view, only limited countries worldwide have a legislation code for pollen manufacturing (Campos *et al.*, 2008). Thus, mush more studies for understanding the microbial load and critical quality control levels for pollen and pollen products are necessary. In this study, 15 samples of marketed pollen were analysed for their microbial content. Isolates of microbial contamination indicators were subjected for identification and antibiotic susceptibility testing.

4.2 MATERIALS AND METHODS

4.2.1 *Sampling*

A total of 15 samples of pollen were collected from local public markets. 13 samples were packed in glass or plastic containers without vacuum and 2 hand-collected other samples were freshly obtained from bee-keepers. All the samples (150 g each) were transported to the laboratory and stored at 4°C, until testing. They were analysed within 24 h of sampling. Prior to analysis, 25 g of each sample was homogenized for 10 min with 225 mL of 0.1% sterile peptone water (10^{-1} dilution). Serial dilutions were performed as required. The pH of the food samples was measured using a digital pH meter (Hanna 8417, Italy) in a 1:10 (w/v) mixture of the homogenate in sterile distilled water. Moisture of each sample was measured after drying three subsamples (1 g each) at 95°C until the obtention of constant weight.

4.2.2 *Total aerobic plat counts:*

The pollen samples of which dilution has been prepared were plated on plate count agar (PCA). Plates were incubated at 30°C for 72 hours aerobically.

4.2.3 *Enumeration and identification of molds*

From each dilution in peptone water, 0.1 mL was spread onto potatoes Dextrose Agar (PDA). The plates were incubated at 25°C for 5 days. Each distinct mould colony was observed microscopically for morphological characterization and identification (Domsch *et al*., 1980; Raper and Fennell, 1965).

4.2.4 *Enumeration of Enterobacteriaceae*

Enterobacteria were counted by transferring a 100 µL aliquot of the appropriate dilution into Petri plates and poured with Mac Conkey agar and Brilliant Green Lactose Bile agar (BGLA). Plates were incubated at 35°C and typical colonies were counted after 24 h of incubation and subjected for biochemical identification.

4.2.5 *Isolation of Salmonella spp.*

Salmonella spp. was detected in 4 steps. Pre-enrichment (25 g of pollen in 225 mL in buffered peptone water) at 37°C for 16-20 h, was followed by enrichment in Rappaport-Vassiliadis (RV) broth incubated at 42°C for 24 h. The isolation was done on xylose lysine desoxycholate (XLD) agar at 37°C for 24 h. The colonies on the XLD agar plate were transferred to a triple sugar iron agar slant (TSI) and incubated at 35°C for 24 h. The colonies on the TSI agar slant were chosen for identification based on morphological and biochemical tests.

4.2.6 Isolation of Staphylococcus aureus

Enrichment of the bacteria was done by adding one gram (1g) of the sample into peptone water and incubated for 18 h at 37°C. Isolation of the *Staphylococcus aureus* was achieved by streaking the pre-enriched culture from the peptone water on to a selective differential agar plate of Baird-Parker Agar (BPA) which was freshly prepared following manufacturer's instructions. The plates were then incubated at 37°C for 24 h under aerobic conditions. Suspected colonies of being *S. aureus* (circular, smooth, convex, moist, and gray to jet-black, frequently associated with an outer clear zone) were subjected to biochemical tests.

4.2.7 *Detection of Listeria* spp.

Twenty-five grams of pollen was homogenized in 225 mL of Listeria-Enrichment Broth (LEB) for 48 h at 37°C. Loopful of culture was streaked on to Listeria-Selective Agar (LSA). Characteristic positive colonies were picked up and subcultured in Brain Heart Infusion Broth (BHI) at 37°C for 24 h and conserved in the same broth containing glycerol (15%, v/v) at 5°C for further biochemical confirmation.

4.3 RESULTS

4.3.1 *Moisture and pH*

Fifteenth pollen samples purchased from local Algerian markets were subjected for microbiological analysis. Moisture and acidity (pH) of the samples were also measured (Table 11). However, pH values varied from 4.55 (from Mila sample) to 6.29 (from Egypt and Biskra samples). Statistical analyses indicate that there was no significant difference between pH values of analysed pollen samples ($P > 0.05$). In addition, moisture content of pollens ranged from 18.11% to 36.29% (samples from Egypt and from Constantine respectively). Raw pollen obtained from Biskra and Constantine has the highest moisture content (30.36 and 36.29% respectively, Table 11). A two-way ANOVA test revealed that there was a significant difference ($P < 0.05$) between relative humidity of pollen samples PS-Alg1/PS-Egy, PS-Alg2/PS-Cons, PS- Chi1/PS-Egy, PS-Alg3/PS-Cons, PS-Alg4/PS-Cons, PS-Bli/PS-Cons, PS-Msi/PS-Cons, PS-Bis/PS-Egy, PS-BBA/PS-Cons, PS-Syr/PS-Egy, and PS-Egy/PS-Chi2. Furthermore, extremely significant difference between moisture of pollen from Egypt and Constantine ($P < 0.001$) was observed.

4.3.2 *Total Microbial Load*

Total microbial count indicates that all pollen samples with the exception of pollen from Egypt, second sample from China and sample from Constantine (CFU=0), have less or more microbial load (Log CFU/g = 3.00 for pollen from Syria to Log CFU/g = 5.48 for pollen from Blida, Table 11). Furthermore, height fungal loads was observed for the pollen sample from Biskra and from Egypt (Log UFC/g = 6.92 and 6.99 respectively, Table 11), a middle fungal content (Log UFC/g=2.3 to 4.48) for the other samples. Statistical analysis (Two-way ANOVA test) indicates that there were no significant differences between values total microbial count as well as fungal count for all pollen samples ($P > 0.05$).

4.3.3 *Pathogen Content*

The absence of *Staphylococcus aureus*, was noticed only in one pollen sample imported from China. The bacterial density of the other samples varied from 2.30 to 8.32 Log CFU/g. The highest was for pollen imported from Egypt (8.32 Log CFU/g) followed by 7.89 Log CFU/g for pollen from Biskra (Table 11). The average of *Staphylococcus aureus* density for the other samples ranged from 2 to 4 Log CFU/g (Table 11).

Table 11: Moisture, pH and microbial count for 15 pollen samples. Results are expressed as Log CFU/g of wet pollen and presence (+) or absence (-) for *Salmonella* and *Listeria*.

Pollen samples	TAMC[a]	TMYC[b]	*Staphylococcus aureus*	Enterobacteria	*Salmonella* spp.	*Listeria* spp.	pH	Moisture (%)
PS-Alg1	3.30	2.770	2.477	7.18	+	+	4.72	28.42
PS-Alg2	3.30	2.690	2.903	ND	-	-	5.11	25.79
PS-Chi1	3.60	3.450	ND	ND	-	+	6.03	27.92
PS-Sét	3.95	3.230	3.301	8.016	+	-	5.86	26.85
PS-Alg3	3.85	2.300	2.903	5.38	+	+	4.97	25.43
PS-Alg4	3.60	2.300	2.903	6.96	+	-	5.33	25.48
PS-Bli	5.48	2.850	2.602	5.72	+	+	5.18	26.58
PS-Mil	4.56	4.090	3.204	ND	-	+	4.55	27.57
PS-Msi	3.70	2.480	2.301	ND	-	+	5.30	25.14
PS-Bis	5.49	6.920	7.890	4.18	+	+	6.30	30.36
PS-BBA	3.30	3.080	2.477	ND	-	+	5.19	26.18
PS-Syr	3.57	3.000	2.477	4.32	-	-	5.51	28.36
PS-Egy	ND[c]	6.990	8.320	7.67	+	+	6.29	18.11
PS-Chi2	ND	4.480	2.778	4.20	-	-	6.14	27.90
PS-Cons	ND	4.210	6.420	6.41	-	+	5.38	36.29
Safety criteria (Campos *et al*. 2008)	< 5 Log CFU/g	< 4.7 Log CFU/g	Absent/1 g	< 2 Log CFU/g	Absent/10 g	/	/	/

[a] TAMC. Total aerobic mesophylic count; [b] TMYC. total molds and yeasts count; [c] ND. not detected; +. presence; -. absence.

PS, pollen sample; Alg, Algiers; Chi, China ; Sét , sétif; Bli, Blida ; Mil, Mila ; Msi, M'Sila ; Bis, Biskra ; BBA, Bordj Bouarirridj; Syr, Syria; Egy, Egypt; Cons, Constantine.

Table12: Fungal species isolated from pollen samples.

Molds	Samples														
	PS-Alg1	PS-Alg2	PS-Chi1	PS-Sét	PS-Alg3	PS-Alg4	PS-Bli	PS-Mil	PS-Msi	PS-Bis	PS-BBA	PS-Syr	PS-Egy	PS-Chi2	PS-Cons
Aspergillus flavus		+							+		+	+			
A. niger	+		+								+	+			
A. alliaceus			+	+										+	+
Penicillium sp. 01		+				+				+					
Penicillium sp. 02		+			+								+		
Penicillium sp. 03				+											
Penicillium sp. 04				+											
Penicillium sp. 05					+										
Penicillium sp. 06				+											
Alternaria alternata				+				+						+	
Alternaria sp. 01				+			+								
Alternaria sp. 02				+			+								+
Monila sitophilia							+								
Cladosporium werneckii				+								+			
Drechslera tritici-repentis				+											
Verticillium albo-atrum	+				+				+				+	+	
Rhisomucor pusillus	+				+										
Mucor hiemalis					+										
Sepedonium chrysospermum				+				+	+						
Phialophora verrucosa	+	+				+	+	+						+	
Monascus ruber								+							
Geotrichum candidum							+								+

+, present; PS, pollen sample; Alg, Algiers; Chi, China ; Sét , sétif; Bli, Blida ; Mil, Mila ; Msi, M'Sila ; Bis, Biskra ; BBA, Bordj Bouarirridj; Syr, Syria; Egy, Egypt; Cons, Constantine.

A significant statistical difference ($P < 0.05$) exists between Log CFU of one sample imported from China and pollen sample from Biskra. However, a very significant difference ($P < 0.01$) was also observed between values the same chine's pollen sample and that for the sample imported from Egypt. No statistical difference was observed for values of enterobacteria count ($P > 0.05$). The average of enterobacteria count ranged from 4.18 to 8.016 Log CFU/g for ten samples. In opposite, counts of enterobacteria for the other five samples were negative. It seems that samples from Sétif, Egypt, Constantine, Blida and Algiers were very contaminated (5.38 to 8.016 Log CFU/g). As another indicator of fecal contamination, *Salmonella* was recovered from seven samples (Table 11). Elevated bacterial density was observed. Only one sample of imported pollens (from Egypt) contains *Salmonella* spp., the other analyzed samples which contain these bacteria

were locally produced. Also, *Listeria* was detected in 10 samples, from which 2 were imported, one from China and another from Egypt (Table 11).

4.3.4 *Microbial identification*

Twenty-two fungal isolates were assigned to species level based on morphological characteristics. They were characterized to 16 different species (*Aspergillus flavus, A. niger, A. alliaceus, Penicillium* sp., *Alternaria alternate, Alternaria* sp., *Monila sitophilia, Cladosporium werneckii, Drechslera tritici-repentis, Verticillium albo-atrum, Rhisomucor pusillus, Mucor hiemalis, Sepedonium chrysospermum, Phialophora verrucosa, Monascus ruber* and *Geotrichum candidum*) (Table 12). Furthermore, From Mac Conkey agar plats, several and morphological different colonies derived from the culture of each pollen sample were subjected for biochemical characterization. Table 13 shows results of the identified isolates belonging to enterobacteria.

Table13: Bacterial isolates belonging to enterobacteria recovered from pollen samples.

Number of isolats	Pathogen identity	Pollen samples in which pathogen was recovred
12	*Salmonella* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét
25	*Shigella* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1
30	*Proteus mirabilis*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
28	*Citrobacter diversus*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
14	*Klebsiella* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
26	*Escherichia coli*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
19	*Providencia* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Chi2, PS-Mil, PS-Msi
29	*Enterobacter cloacae*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2

4.4 DISCUSSION

Plant products have been identified and confirmed as a significant source of pathogens and chemical contaminants that pose a potential threat to human health worldwide. There has been a growing interest in developing and applying microbiological criteria to the sanitary control of beehive products especially pollen. Ideally such standards should be based on bacterial counts associated with disease. Instead, a standard related to sanitation practices might specify the permissible number of microorganisms in an acceptable food product derived from CFU counts obtained in a large-scale study on representative pollen samples. Usefulness of these study data is limited since the study was designed to survey microbial counts in only fewer samples.

In the present study, 15 pollen samples were analysed. Their acidity, moisture, microbial load and pathogen content were determined. Enormous total aerobic mesophylic count (TAMC) was recorded for 12 samples (>3 Log CFU/g). In three samples total aerobic mesophylic microorganisms was nil even their pH values were near neutrality and they have low moisture. It seems that microorganisms in these samples were in dormant state and needs longer period than that of incubation time for adaptation to the laboratory growth conditions. Furthermore, total molds and yeast count (TMYC) was also important. All pollen samples have remarkable fungal density. Comparing our results with that recommended by Campos *et al*. (2008) (TAMC not exceed than 5 Log CFU/g and a TMYC less than 4.7 Log CFU/g), three pollen samples (PS-Bli, PS-Bis, and PS-Egy) were out of safety recommendations. In addition, according to the same safety criteria (Absent of *Staphylococcus*/1 g and enterobacteria should be less than 2 Log CFU/g); all pollen samples (except that imported from China, PS-Chi1) were of pour microbiological quality. Ten samples contain more than 2 Log CFU of enterobacteria/g, and 14 samples contain more than 8 Log CFU of staphylococci per gram of pollen. More importantly, the detection of *Salmonella* in seven pollen samples is a direct evidence for a fecal contamination, and renders the food material a potentially product and indicates poor food handling practices.

In fact, the test for *Enterobacteriaceae* has replaced the tests for coliforms that traditionally have been used as indicators of hygiene and contamination after processing. The major problems with the coliform tests are the variability in definition of the term coliforms (they are defined usually by the method used for their detection) and the fact that only lactose fermenting organisms are detected. In comparison the family *Enterobacteriaceae* is well defined taxonomically and methods for their enumeration are based on common properties. Furthermore, the methods also detect important non-lactose fermenting organisms such as *Salmonellas* (Gilbert *et al.*, 2000). Also, *Listeria* spp. other than *L. monocytogenes* are rarely implicated in illness. They are indicators for the likely presence of *L. monocytogenes.*

Furthermore, this pathogen is widely distributed in the environment and is able to multiply slowly at 4°C. The shelf life of foods varies enormously, and the presence of *L. monocytogenes* at any level may be of significance due to its potential for growth during storage. The use of an enrichment procedure, in addition to enumeration, should therefore be considered to ensure that the organism is absent from the product.

From another point of view, the slightly acidic aspect of the analyzed pollen samples favors the development of fungi and mycotoxin production. *Aspergillus, Penicillium, Alternaria, Mucor* and others were recovered from pollen. They are involved in various diseases such as allergic illness, mycotoxicosis, and aflatoxicosis (Martin, 1974). In addition, the recovery of potent pathogenic bacteria belonging to the family *Enterobacteriaceae* in another proof of unsatisfactory and unacceptable pollens set for human consumption in local markets. A great attention should be taken developing legislation considering pollen and pollen extracts as well as standards for microbial quality control of this type of products. More importantly, expanded studies about pollen processing and preservation to reduce microbial load pathogen elimination are necessary.

Chapter 05

Population dynamics of Lactic acid bacterial

5. Population Dynamics of LAB During Laboratory Fermentation of Honeybee- Collected Pollen.

5.1 INTRODUCTION

Traditionally lactic acid bacteria (LAB) have been considered to include low G+C content Gram-positive bacteria included in the phylum Firmicutes that are used as starters for industrial food fermentations, notably those based on raw materials derived from milk, meat and plants. Plants, foods, fermented products, animals and humans constitute natural ecological systems and good sources for LAB. Flowers and their constituents are parts of the plant phylloplane. Stirling and Whittenburg (1963) suggested that the LAB are not usually part of the normal microflora of the growing plant and indicated the role of insects in the spread of these organisms. LAB commonly found on fresh herbage and in silage have been investigated (Weise, 1973; Woolford, 1975). Nilsson and Nilsson (1956) found that the predominant LAB during silage fermentation were streptococci and lactobacilli, with *Lactobacillus plantarum* the species most frequently recovered. Other studies (Mundt and Hammer, 1968; Mundt *et al.*, 1969) reported the occurrence of pediococci and lactobacilli on leaving or decayed plants. Lactobacilli commonly share the habitat phyllosphere with species of the genera *Leuconostoc*, *Pediococcus* and *Weissella*. Species frequently recovered from the leaves include *Lb. plantarum*, *Lb. paracasei*, *Lb. fermentum*, *Lb. brevis* and *Lb. buchneri* (Hammes and Hertel, 2006). Part of the accumulated information about the occurrence of LAB members on plants is derived from microbiological studies of the fermentation process. Thus, the microbial population upon initiation of the process is known for several plants (grasses, cabbage, silage raw materials, carrots and beets, olives and fruits such as grapes and pears, etc.). But scarce information about the occurrence of LAB on flowers and pollens is available in the literature.

In this investigation, population dynamics of some LAB groups or genera was addressed after selective enrichment-based fermentation of raw pollen grains. Variation of acidity was also determined.

5.2 MATERIALS AND METHODS

5.2.1 *Enrichment and enumeration of LAB.*

Two pollen samples (polyfloral pollen and palm pollen) were diluted (20%, w/v) in Elliker broth pH 6.5 (Elliker *et al.*, 1956). The pH of the mixture was adjusted to 7.02 using sterile 0.1 N HCl or 1 N NaOH. The cultures were incubated anaerobically at 30°C for 72 hr under agitation (100 rpm). At six hours intervals, four LAB groups were counted on selective solid media. From each enrichement culture, serial dilutions were prepared as needed in peptone water (0.1 % peptone and 0.1 % of Tween 80), and aliquots (100 µL) from an appropriate dilution were cultured on the following media: M17 agar (Fluka) incubated for 72 hr at 30°C for lactococci and streptococci, LBS agar (Rogosa *et al.*, 1953) after anaerobic incubation (BBL GasPack System) at 30°C for 72 hr for lactobacilli, Glucose Yeast Extract Agar (Holzapfel *et al.*, 2006) incubated at 30°C for 48 hr to isolate leuconostocs and pediococci, and Enterococcus agar, according

to Slanetz and Bartley (Merck), incubated at 30°C for 48 hr to isolate enterococci. After incubation, appeared colonie were counted and bacterial density was expressed as Log_{10} CFU/mL. Changes of culture pH were also measured.

5.3 RESULTS

Figure 10 shows the course of pH and the different LAB groups selected for poly-floral-pollen (A) and palm pollen (B). In general, simultaneous growth of enterococci, lactobacilli, streptococci/lactococci, and pediococci/leuconostocs took place; no other major groups of microorganisms were involved, except the occurrence of yeasts at the end of the fermentation of palm pollen (data not shown). Enterococci counts of 0.21 to 5.03 Log CFU/mL of poly-floral pollen (Fig. 10A) were present at the beginning and the end of the fermentation. The bacterial populations size of pediococci/leuconostocs, lactococci/ streptococci, and enterococci increased during the first 6 to 12 h and grew to a maximum population of 6.20 to 6.80 log CFU/mL, whereas, lactobacilli occur after 11 h of the fermentation and reached 7.54 Log CFU/mL. Upon prolonged fermentation (after 30 h), population of all groups except lactobacilli declined to 5.03 to 5.40 log CFU/mL. The lactobacilli continuous to increase until 36 h, sometimes stabilizing upon prolonged fermentation (7.42 to 7.45 Log CFU/mL). After 54 h, they declined to reache 6.20 Log CFU/mL at the end of the fermentation (Fig. 10A). All studied LAB groups during the fermentation of the poly-floral pollen have similar growth pattern upon their occurrence. Two phases characterizes the growth curve. An exponential phase during the first 36 h, and a stationary phase during the last 36 h. a simultaneous growth was observed and same population dynamic was shared by studied LAB groups. Considering pH development, however, three phases appears during the fermentation. A drastic shift during the first 18 h reaching 3.84 followed by a slight increase to 4.56 after 12 h, and then slowly decreased to a minimum level of 3.46 (Fig. 10A). The shift phase occurs during the beginning of the exponential phase of bacterial growth, and the second phase persists for the last period of the exponential phase, followed by a slow decrease during the stationary phase. In comparison to the fermentation of poly-floral pollen, development of LAB groups during spontaneous fermentation of mono-floral pollen appears to share the same pattern as it is for the LAB population in the poly-floral pollen (Fig. 10B). A first phase, characterized by the increase of LAB density, except for lactobacilli, which appeared only after 18 h, followed in general by a remarkable increase of bacterial density, and then the growth entered to a stationary phase. *Enterococcus* reached the highest concentration (7.5 Log CFU/mL) after 30 h, which was accompanied by a slight decrease in the population of streptococci/lactococci and a shift in pH of the solution to its minimal value, 3.94 (Fig. 10B). The population size of the enterococci decreased drastically by 2.1 Log orders after 36 h of the fermentation, accompanied by reaching lactobacilli, and pediococcus/leuconostocs their maximum levels (6.86 and 7.2 Log CFU/mL, respectively). For a prolonged fermentation period (after 42 h), LAB densities persist with a slight decrease (3.83 to 4.35 Log CFU/mL, for enterococci and streptococci/lactococci, respectively; and 5.2

Log CFU/mL for lactobacilli). Pediococci/ leuconostocs group appeared to reach the highest concentration at the end of the fermentation (6.55 Log CFU/mL), however, the pH persist for the last 18 h near 4 (Fig. 10B).

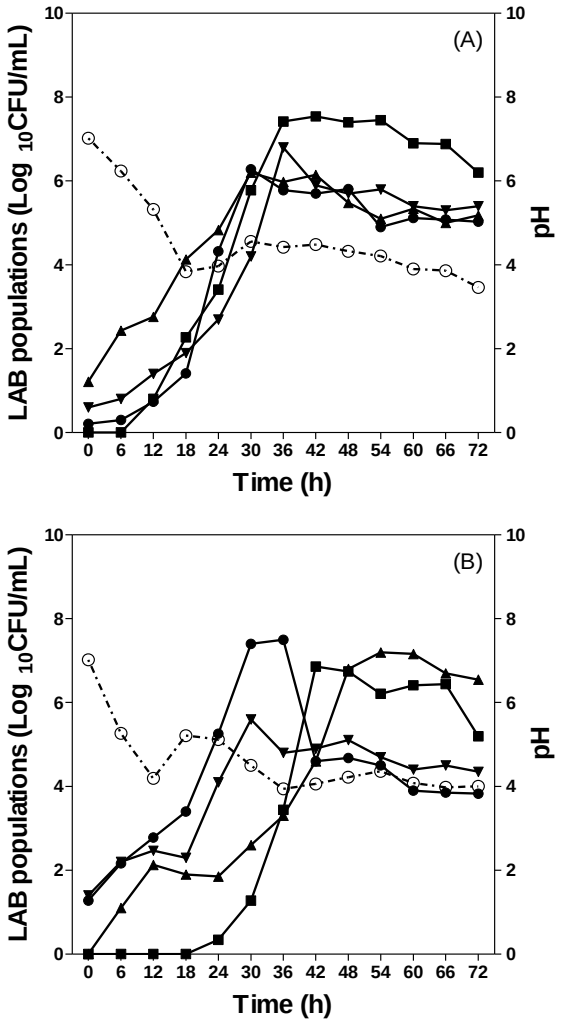


Figure10: LAB populations during fermentation of poly-floral pollen (A) and date palm pollen (B). Microbial succession (Log CFU/mL) of enterococci (-●-), lactobacilli (-■-), pediococci and leuconostocs (-▲-), streptococci and lactococci (-▼-), Culture pH (-○-) variation during 72 hour of incubation.

5.4 DISCUSSION

Fermentation of pollen grains was addressed only by Pain and Maugenet (1966). They sterilized pollen by Gama-irradiation and inoculated it by *lactobacillus* starter culture. The resulting fermented material has unappetizing product and of poor nutritive value. Instead of that, however, no studies exploring the self fermentation of pollen at the laboratory scale exists. After collection by honeybees, pollen is mixed with nectar and salivary secretions from the foraging bees, and compacted in the comb cells followed by the addition of a honey and a waxy layer as coating material. It is postulated that pollen undergoes self fermentation inside comb cells. This fermentation conducted by yeasts, bacteria and molds. Microorganisms responsible of this fermentation are of external origin (from plant materials, nectar, water, honeybees, the comb, etc.) (Belhadj *et al.*, 2012). They represent a complex and undetermined starter (poly-microbial mixed population). In the present work, results indicate that major LAB genera were present during the fermentation, and they share the same growth pattern. A synergistic phenomenon exists during growth. Analyses of pollen chemistry have shown that pollen ranges from 2.5% to 61% protein by dry mass (Buchmann, 1986). Microbial flora of pollen contributes to the hydrolysis of pollen proteins. This effect is achieved by secretion of extracellular hydrolytic enzymes (protease, lipase, cellulase, amylase, etc.) for degradation of pollen proteins and other macromolecular materials (Roulston and Cane, 2000). This degradation resulting in the liberation of free low molecular mass and simple molecules (sugars, amino acids, lipids, etc.), and the biosynthesis of other stimulating growth factors (vitamins, nucleotides) needed by other bacteria. The enterococci and lactococci/streptococci found in pollen with height concentration and they predominates the other LAB genera. In protein riche foods such as meat and fish, they contribute to the liberation of free amino acids needed by lactobacilli and other LAB species. LAB require external nitrogen sources, and as fastidious organisms, it is clear that LAB have adapted to rich environments by developing systems to efficiently exploit the nitrogen sources present there. The essential feature of LAB metabolism is efficient carbohydrate fermentation. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end-product is, of course, lactic acid (>50% of sugar carbon), which decreases pH of the medium in part, and inhibits or decreases the growth of other microorganisms

(Salminen *et al.*, 2004). Antagonism effect can be assured also by the biosynthesis of bacteriocins which affect the development of closely related species. The drastic decrease of bacterial population (lactococci/streptococci, enterococci) accompanied by a shift in pH and the increase of other population (lactobacilli, pediococci/leuconostocs) may be explained by the antagonism effect generated by lactobacilli for example or the competition for nutriments as another mechanism involved natural in poly-microbial systems. It is not clear what are the mechanisms and the species involved in pollen degradation as well as the environmental conditions (pH, temperature, moisture, pollen chemical composition, etc.) conducting to the right fermentation of pollens. Furthermore, other microorganisms (non LAB bacteria and yeasts) which contribute to the self fermentation of pollen should be determined.

Chapter 06

Characterization of Lactic acid bacterial

6. Phenotypic and Genotypic Characterization of Some Lactic Acid Bacteria Isolated From Bee Pollen: A Preliminary study.

6.1 INTRODUCTION

Beehive products (honey, pollen, propolis and royal jelly) are natural functional foods that have gained increased attention in society (Viuda-Martos *et al*., 2008). Pollen, the male gametophyte of flowering plants, is a high-energy material collected by honeybees and other insects and stored as a food reserve. Pollen has been used traditionally by humans as a supplementary food and in alternative medical treatments. It has been used medically in prostatitis, bleeding stomach ulcers and some infectious diseases (Linskens and Jorde, 1997).

Because of its complex content, bee-pollen has a very important nutritional value in the human diet (Gergen *et al*., 2006). Since the middle of the last century, the bee-pollen microflora has been investigated (Chevtchik, 1950; Gilliam, 1979a; 1979b; Gilliam *et al*., 1989; Gilliam *et al*., 1990; Belhadj *et al*., 2010; 2012). However, little is known about the occurrence of lactic acid bacteria (LAB), and the roles they play, in pollens. Only a few reports are available in the literature considering this field. Occurrence of Lactobacilli in pollens was reviewed by Gilliam (Gilliam, 1979a). In another study, Vásquez and Olofsson (2009) identified lactobacilli isolated from pollen grains. These isolates were identified based on PCR and 16S rRNA gene sequencing. Other LAB representative genera (*Lactococcus, Lactobacillus, Pediococcus, Enterococcus* and *Leuconostoc*) were recovered and identified based on phenotypic traits (Belhadj *et al*., 2010). In the latter investigation, *in vitro* studies indicated that several strains inhibit Gram-positive and Gram-negative pathogenic bacteria. Additionally, several members of LAB are known to produce antibacterial substances such as organic acids and bacteriocins. Antagonism towards undesirable microorganisms is an important criterion for LAB being used as bio-preservations or biocontrol agents. It seems that pollens are a suitable ecological niche for various microorganisms and an important source for the isolation of new strains belonging to the LAB group with antagonistic activity against harmful bacteria. Species or subspecies identification of such strains is recommended. In fact, physiological and biochemical criteria used for LAB identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, clear identification to the species level by simple phenotypic tests may sometimes be difficult (Visser *et al*., 1986). Indeed, additional molecular-based characterization approaches such as 16S rRNA gene sequencing, 16S-ARDRA, PFGE and other methods are used for assignment of a given LAB strain to its taxonomic status.

The present work describes isolation and phenotype-based numerical clustering of LAB isolated from pollen grains collected in some Algerian areas; 16S rRNA gene sequencing-based characterization of selected strains was investigated. Also, their antagonistic activity against Gram-positive and Gram-negative bacteria was evaluated.

6.2 MATERIALS AND METHODS

6.2.1 *Isolation of LAB from pollen*

Previous studies (Belhadj *et al*., 2010); indicate that pollen samples taken from pollen traps are highly contaminated, especially by enterococci. Furthermore, dilution-based LAB isolation is not a sufficient manner for the recovery of these bacteria from pollen grains, and full negative results are unavoidable with this method. Hence, a simple technique is used the efficient isolation of LAB. In the field during flowering seasons, honey bee foragers are kinched (catched) by a sterile stainless steel or plastic forceps. The pollen pellets (60 samples from 10 clean regions; six subsamples from each sampling site; in Bordj Bou Arreridj and Sétif) are then collected by a humidified sterile cotton swab by gently touching the pollen pellets attached to the posterior legs of the bee. At this time, we can discuss the facts regarding bee-collected pollen from a microbiological point of view. The swabs were transported to the laboratory at low temperature (5°C) and analyzed or further maintained with refrigeration until use. Because LAB isolation is enrichment-based method, the quantity of pollen recovered is not important. Each swab was then introduced into a capped glass tube containing 15 mL Elliker broth pH 6.5 (Elliker *et al*., 1956), and incubated anaerobically at 30°C for at least two days. Afterwards, serial dilutions were prepared from each tube in peptone water (0.1 % peptone and 0.1 % of Tween 80), and from the appropriate dilution, aliquots (100 µL) were spread and cultured on the following media: M17 agar (Fluka) incubated for 72 hr at 30°C for lactococci and streptococci, LBS agar (Rogosa *et al*., 1953) after anaerobic incubation (BBL GasPack System) at 30°C for 72 hr for lactobacilli, Glucose Yeast Extract Agar (Holzapfel *et al*., 2006) incubated at 30°C for 48 hr to isolate leuconostocs and pediococci, and D-MRS agar (Hammes and Hertel, 2006) incubated at 25°C for 72 hr to isolate carnobacteria. Enterococci were treated in a separate study. From each culture, 10–30 colonies were randomly picked up and further purified on MRS agar (pH 6.5). Pure isolates were maintained at –20°C in MRS broth containing glycerol (20%, v/v final concentration).

6.2.2 *Screening for antagonistic activity*

For detection of antagonistic activity, an agar well diffusion assay was used according to Schillinger and Lucke (1989). Bacterial species used as indicator microorganisms were listed in Table 14. LAB isolates were subcultured twice (1% inocula, 24 hr, 30°C) in 10 mL MRS broth (Fluka). The non-LAB were subcultured twice (1% inoculum, 24 h, 37°C) in 10 mL BHI broth (Fluka) and kept frozen at –20°C in BHI broth supplemented with 20% glycerol. Cell-free supernatants from LAB cultures were obtained by centrifuging the cultures (8000 g/10 min at 4°C), and then the pH of each supernatant was adjusted to 6.5 using 5N HCl followed by filtration through a cellulose acetate filter with a pore size of 0.2 µm. Before supernatant neutralization, an antimicrobial assay was performed for all isolates. All experiments were performed in duplicate, and the results were displayed as the mean value of the experiments.

Isolates showing antagonistic activity against one or more indicator bacteria (with an inhibition zone diameter of more than 5 mm) were subjected for phenotypic characterization.

Table 14. Indicator bacteria used and their growth conditions.

Indicator species	Strain no.	Origin	Growth medium	Incubation conditions
Gram-positive bacteria				
Bacillus subtilis	20302	CLAM[a]	BHI[c]	37°C
Enterococcus faecium	H421	CLAM	MRS[d]	30°C
Listeria innocua	3030	CLAM	BHI	37°C
Staphylococcus aureus	25923	ATCC[b]	BHI	37°C
Gram-negative bacteria				
Escherichia coli	25922	ATCC	BHI	37°C
Salmonella typhimurium	1717	CLAM	BHI	37°C
Pseudomonas aeruginosa	27853	ATCC	BHI	37°C
Shigella sp.	96415	CLAM	BHI	37°C

[a] Collection of Laboratory for Applied Microbiology.
[b] American Type Culture Collection.
[c] Brain heart infusion broth
[d] De Man, Rogosa and Sharpe broth

6.2.3 *Phenotypic identification of bioactive isolates*

Isolates exhibiting antagonistic activity were selected on the basis of Gram staining, morphology, tetrad formation and catalase activity. Catalase-negative and Gram-positive rods and cocci were selected and screened for the production of CO_2 from glucose (in MRS broth, containing Durham inverted tubes, without beef extract and citrate). Ability to grow at 10 and at 45°C was evaluated in MRS broth after incubation for 7 days and 48 h, respectively. Growth in MRS containing 6.5 or 18% NaCl, as well as growth in MRS with pH 4.4 and 9.6, was studied. Acid production from carbohydrates (glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-arabitol, L-arabitol, and gluconate) was evaluated by miniaturized assay in 96-well flat-bottom microtiter plates (Parente *et al*., 2001). Sterile carbohydrate solutions (Institut Pasteur, France) were added to the basal medium (MRS without glucose and meat extract and with 0.16 g/L bromocresol purple, pH 7.0) at a final concentration of 1%. Cells were harvested from overnight cultures by centrifugation (10,000 g, 5 min, 4°C), washed twice in sterile phosphate buffer (pH 7.0) and suspended in sterile saline (0.85% NaCl). This

suspension was used to inoculate sterile microtiter plates (96 flat-bottom wells), which were incubated in anaerobiosis for 7 days at 30°C. Esculin hydrolysis was assessed by adding 2 g/L esculin (Sigma) and 5 g/L ferric ammonium citrate (Sigma) to the basal medium.

Fermentation of each of the 39 carbohydrates was interpreted as follows: positive (+), complete change to yellow; weakly positive (w), change to green; and negative (-), no change at all. Esculin hydrolysis (revealed by a change to a darker color) was interpreted as positive (+), while no change was negative (-). Strains were tested in duplicate to determine the test reproducibility.

6.2.4 *Genotypic identification of selected LAB strains*

Sample preparation prior to PCR amplification

As described by Rodas *et al.* (2003), selected LAB isolates (10 strains displaying a remarkable assimilatory pattern) were grown in MRS agar at 30°C for 2 days. One single colony was picked up from plates and suspended in 20 µL of sterile distilled water. These suspensions were used for PCR reactions without further processing.

Amplification and sequencing of 16S rRNA gene

The protocol of Rodas *et al.* (2003) was used for 16S rRNA gene amplification using primers pA (8-AGAGTTTGATCCTGGCTCAG-28) and pH (1542-AAGAGGTGATCCAGCCGCA-1522) (Edwards *et al*., 1989). DNA amplification was carried out in a 50 µL PCR mixture containing 200 µM dNTP, 1 µM of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 6 U of *Taq* DNA polymerase and 1 µL of the cell suspension. Each PCR cycle consisted of an initial denaturation time of 5 min at 94°C followed by 35 cycles of amplification comprising a denaturation step for 30 sec at 94°C, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. Reactions were completed with 5 min elongation at 72°C followed by cooling to 10°C. PCR products were resolved by electrophoresis at a constant voltage (200 V) in 1.2% (w/v) agarose in 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA pH 8.0) gels stained with ethidium bromide (0.5 µg/mL). Amplification products were purified using a PCR purification kit (Qiagen, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and an automated DNA sequencer (ABI Prism® 3100 Avant Genetic Analyzer, Applied Biosystems). The nucleotide sequences of the 16S rRNA gene of all the isolates were analyzed and determined by the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov/). The alignments were analyzed to construct a phylogenetic tree and to compare similarities among the sequences by the neighbor-joining method (Saitou and Nei, 1987) using MEGA software version 4.0. Bootstrap analysis was used to evaluate the tree topology of the data by performing 1000 resamplings. The sequences were deposited in the GenBank database using the web-based data submission tool Sequin (http://www.ncbi.nlm.nih.gov/ Sequin).

6.2.5 *Statistical analysis*

Hierarchical cluster analysis was carried out with Statistica 6 software (Statsoft Italia, Padua, Italy). The Euclidean distance, unweighted pair group method with arithmetic mean (UPGMA) and an index of similarity were used for the analysis of carbohydrate fermentation. A two-way ANOVA test was used for comparing antimicrobial activity of LAB.

6.3 RESULTS

6.3.1 *Isolation and antagonism among LAB strains*

From the ten sampling zones, distributed across two provinces, six samples were collected from each zone. However, a total of 567 isolates were recovered from pollen grains. Growth of yeasts and molds on isolation agar media was observed and confirmed by microscopic examination. Also, catalase-positive bacterial colonies were encountered. Presumptive LAB cells were carefully selected based on catalase reaction, cell shape, motility and Gram staining. All Gram-positive, catalase-negative cocci and rods were purified on suitable agar media to homogeneity. The 567 pure LAB strains were screened for their antagonistic activity by agar well diffusion assay against eight undesirable bacteria belonging to Gram-positive and Gram-negative ranks (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CLAM 20302, *Enterococcus faecium* CLAM H421, *Listeria innocua* CLAM 3030, *Shigella* sp. CLAM 96415, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CLAM 1717, and *Pseudomonas aeruginosa* ATCC 27853). After a random screening process, using neutralized supernatant cultures, 216 stains found to potentially inhibit at least one indicator bacteria, whereas 351 were not. Statistical analysis indicated that total percentage of potent strains (40.74 ± 6.31) was significantly different ($P = 0.003 < 0.05$) from that of non-potent strains (59.26 ± 6.31). From all sampling zones, except zones Sz48 (62.06%) and Sz54 (80.00%), the percentage of antagonistic LAB strains was below 46 (Fig. 11).

The 216 LAB strains showing inhibition zones against at least one indicator bacterium (without pH neutralization of spent cultures), according to the first antimicrobial screening assay, were further subjected to determination of the antibacterial activity of neutralized cell-free supernatants obtained by centrifugation.

Neutralization of culture supernatant pH (6.5) eliminates the effect of acidity on the target bacteria. Furthermore, incubation of LAB under anaerobic conditions minimizes hydrogen peroxide production. Following the second screening assay using eight indicator bacteria (four Gram negative and four Gram positive), 54 potent LAB strains were obtained (Fig. 12).

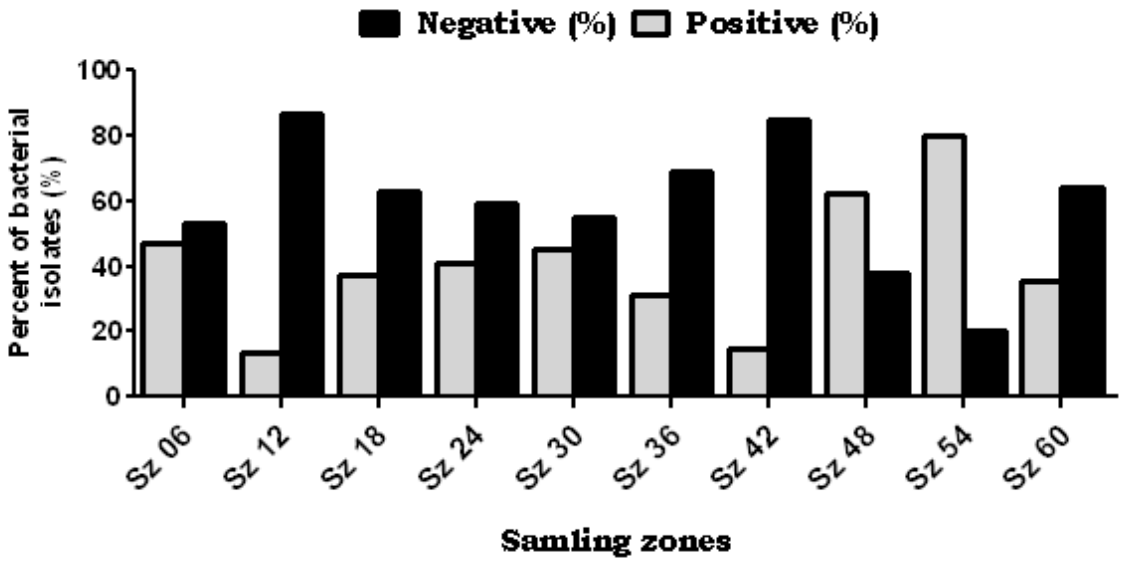


Figure 11: Percent distribution of antagonism among 567 LAB strains isolated from pollen samples. Samples Sz06, Sz18, Sz30, Sz36 and Sz54 were from BBA, whereas samples Sz12, Sz24, Sz42, Sz48 and Sz60 were from Sétif. Statistical analysis indicates that the total percentage of potent strains (40.74 ± 6.31) is significantly different ($P = 0.003 < 0.05$) from that of non-potent strains (59.26 ± 6.31). Percent distributions of potent strains were extremely different from each other ($P < 0.0001$) except for sampling zones Sz36 and Sz60 ($P < 0.01$). Also, there were no statistical differences between the percentage of potent strains of sampling zones Sz06 and Sz30 ($P > 0.05$) or sampling zones Sz18 and Sz60 ($P > 0.05$). Percent distributions of non-potent strains among sampling zones were significantly different from each other ($P < 0.05$) except for sampling zones Sz06 and Sz30, Sz12 and Sz42 and Sz18 and Sz24 and Sz18 and Sz60, which do not differ statistically ($P > 0.05$).

Figure 12A. presents the variation in the antagonistic activity against Gram-negative human and animal pathogenic bacteria. The activity was measured from the zone of inhibition (mm) around the well. This quantified activity varied from 10 to 27 mm for the majority of LAB strains studied (Fig. 12). Furthermore, only two strains (strains 43 and 54) could not inhibit *E. coli* ATCC 25922, two other strains (strains 30 and 36) were inactive against *Salmonella typhi*murium CLAM 1717, and strain 46 was inactive against *Shigella* sp. CLAM 96415 (Fig. 12A). In addition, only five strains were able to produce a zone of inhibition in between 29 and 35 mm against indicator strains, for example, strains 12 and 41 against *E. coli* ATCC 25922 and strains 14, 46 and 47 against *Salmonella typhimurium* CLAM 1717 (Fig. 12A). However, it was observed that *Pseudomonas aeruginosa* ATCC 27853 resisted the inhibitory potential as compared with the other indicator bacteria (Fig. 12A). For Gram-positive indicator bacteria, the majority of inhibition diameters were between 10 and 20 mm for *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* CLAM 20302 and between 10 and 30 mm for *Enterococcus faecium* CLAM H421 and *Listeria innocua* CLAM 3030 (Fig. 12B). However, 14 strains were inactive against indicator bacteria. Strains 5, 21, 22, 37, 43 and 49 were inactive against *Bacillus subtilis;* strains 16 and 22 were inactive against *Staphylococcus aureus;* strains 34, 35, 36 and 38 were inactive against *Listeria innocua;* and strains 5 and 37 were inactive against *Enterococcus faecium.* In addition, it was noted that *Listeria innocua and Enterococcus faecium* were more susceptible to the action of LAB strains than *Bacillus subtilis* and *Staphylococcus aureus.* For the latter bacterium, the inhibition diameters were between 10 and 33 mm (Fig. 12B).

6.3.2 *Clustering structure and analysis*

Fifty-four bacteriocin-like producing isolates of LAB obtained from different raw pollen samples were characterized according to the method of Axelsson (2004), Bergey's Manual (Sneath *et al*., 1986) and the prokaryotes (Dworkin *et al*., 2006). Preliminary, the diversity was studied based on a phenotypic approach. Microplates containing 39 different carbon sources and 10 other physiological traits were used to determine the phenotypic profiles of the 54 isolates of LAB. The reproducibility of the fermentation tests was 100%. The similarity coefficient cluster analysis resulted in five major clusters (A, C, E, F and I, containing three or more strains) defined at the 79.0% similarity level (Fig. 13). The following four other minor clusters were defined at the 79% similarity level (B, D, G and H, two 2-member cluster and two 1-member clusters).

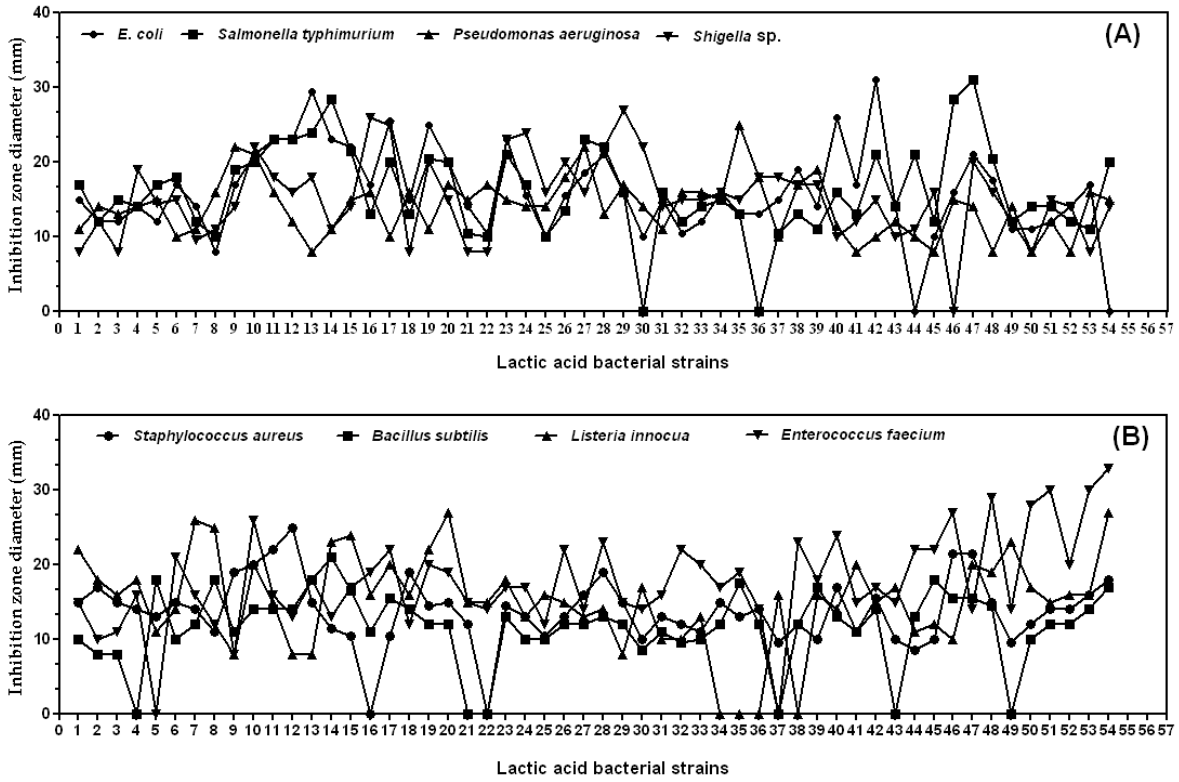


Figure 12: Antibacterial activity (inhibition zone diameter, mm) of 54 pollen residing lactic acid bacterial strains against Gram-negative and Gram positive indicator bacteria (A) *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CLAM 1717, *Pseudomonas aeruginosa* ATCC 27853 and *Shigella* sp. CLAM 96415. Significant statistical differences were observed ($P = 0.0071$, $P < 0.05$) among antimicrobial potential of LAB strains, whereas indicator pathogenic bacteria themselves could not affect the variation in antimicrobial activity ($P = 0.1033$, $P > 0.05$) as determined by two-way ANOVA test. (B) *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CLAM 20302, *Enterococcus faecium* CLAM H421, and *Listeria innocua* CLAM 3030. Two-way ANOVA analysis indicated that there were no significant statistical differences among the antimicrobial actions of LAB strains ($P > 0.05$), but is resulted from the indicator pathogenic bacteria themselves and extremely significant differences were obtained ($P < 0.0001$).

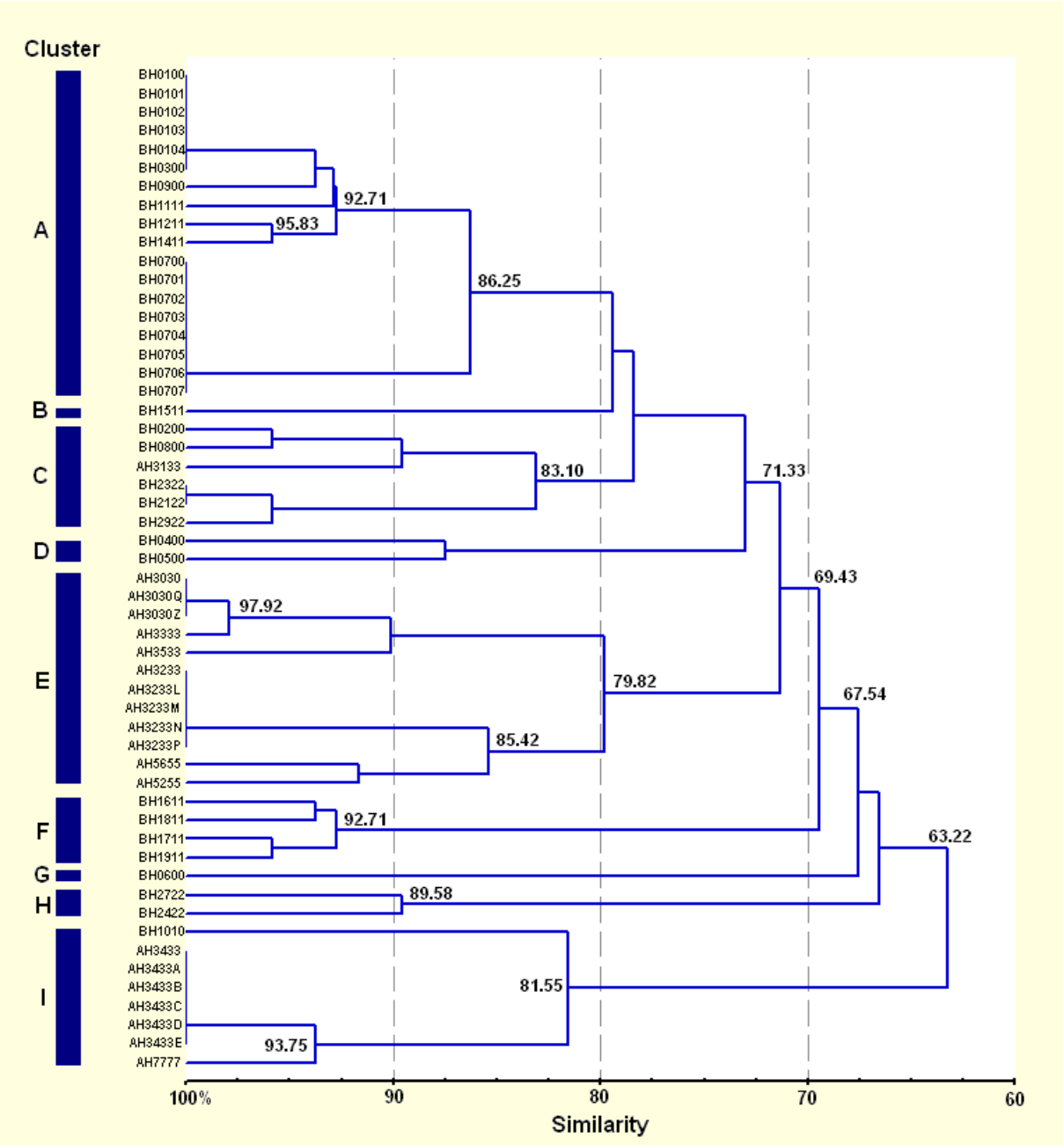


Figure 13: Dendrogram of combined phenotype profiles of 54 potent antagonistic lactic acid bacteria isolates as determined by carbohydrate fermentation and physiologic traits. Cluster analysis was carried out based on the simple matching coefficient and unweighted pair group algorithm with arithmetic averages (UPGMA). Codes of strains and clusters are indicated on the left hand side of the figure.

6.3.3 *Major clusters (A, C, E, F and I)*

Cluster A), Lactobacillus plantarum, Lactobacillus paraplantarum and Lactobacillus sp., comprised 18 isolates from Bordj Bou Arreridj (BBA) (33.33% of the total isolates). The cluster contained eight other *Lactobacillus plantarum* strains, one strain of *Lactobacillus paraplantarum* and eight *Lactobacillus* sp. strains. All strains were homofermentative, and 55.5% of the isolates in this cluster fermented L-arabinose. They did not ferment xylose, adonitol, β-methyl-xyloside, starch, arabitol and rhamnose. Alpha-methyl-D-mannoside was variously fermented (Table 15). The same pattern was noted for gluconate, lactose and sorbitol. Members of this cluster grew at 10 and 45°C in the presence of 6.5% NaCl and at pH 4.4.

Table 15: Differenciatial carbohydrates fermentation by *Lactobacillus* strains belonging to Cluster A.

	Lactobacillus plantarum BH0100, BH0102, BH0103, BH0104, BH0101, BH0300	*Lactobacillus plantarum* BH0900	*Lactobacillus plantarum* BH1111	*Lactobacillus paraplantarum* BH1211	*Lactobacillus plantarum* BH1411	*Lactobacillus sp.* BH0700, BH0700, BH0700, BH0700, BH0700, BH0700, BH0700, BH0700
Glycerol	-	+	-	-	-	+
D-Arabinose	-	-	-	-	+	-
Ribose	+	+	+	+	+	-
D-Xylose	-	+	-	-	-	-
Sorbitol	+	+	+	+	-	-
α-Methyl-D-mannoside	+	-	+	-	-	-
Salicin	+	+	+	+	+	-
Lactose	+	+	+	+	+	-
Innulin	+	+	-	-	-	+
D-Turanose	-	-	+	-	-	-
L-Fucose	+	+	-	-	-	+
D-Arabitol	-	-	+	-	-	-
Gluconate	+	+	-	-	+	-

+, Positive reaction; -, N gative reaction

Table 16: Differenciatial carbohydrates fermentation by *Lactobacillus* and *Lactococcus* strains belonging to Cluster C.

	Lactobacillus plantarum TDI 1777 BH0200	Lactobacillus plantarum BH0800	*Lctococcus lactis ssp lactis* AH3133	*Lactobacillus sp.* BH2322, BH2122	*Lactobacillus sp.* BH2922
Glycerol	-	-	-	+	+
L-Arabinose	+	-	-	-	+
Rhamnose	-	-	-	+	+
Dulcitol	-	-	-	-	-
Inositol	-	-	-	+	-
Mannitol	+	+	-	+	+
Sorbitol	+	+	+	+	+
α-Methyl-D-mannoside	+	+	-	-	+
Amygdalin	+	+	-	+	+
Melibiose	-	+	-	+	+
Melezitose	+	+	-	+	+
D-Raffinose	-	-	+	+	+
D-Arabitol	-	-	-	+	+
Gluconate	-	-	w	+	+
5-Ceto-gluconate	-	-	-	-	+

+, Positive reaction; -, Negative reaction

Cluster C), Lactobacillus plantarum, Lactococcus lactis subsp. *lactis, and Lactobacillus* sp., contained three *Lactobacillus* sp. members, one strain of *Lactococcus lactis* subsp. *lactis* and two *Lactobacillus plantarum* strains. All were homofermentative and grew well at 10 and 45°C, in the presence of 6.5% NaCl and under alkaline conditions (pH, 9.6). They all fermented ribose, hexoses, cellobiose, lactose, saccharose, trehalose and B-gentiobiose, but they did not ferment erythritol, D-arabinose, xylose, adonitol, β-methyl-xyloside, dulcitol, α-methyl-D-glucoside, starch, xylitol, L-arabitol, D-lyxose, D-tagatose, D-fucose and L-fucose. They variously fermented L-arabinose, rhamnose, inositol, mannitol, sorbitol, α-methyl-D-mannoside, melibiose, melezitose, D-raffinose, D-arabitol, gluconate and amygdalin (Table 16).

Cluster E), Lactococcus lactis, Pediococcus pentsaceus and Pediococcus acidilactici, contained five strains of *Lactococcus lactis* subsp. *lactis* (AH3030**,** AH3031**,** AH3032, AH3333 and AH3533), six strains of *Pediococcus pentosaceus* (AH3233**,** AH3233 L**,** AH3233 M**,** AH3233 N**,** AH3233 and AH5655), and one strain of *Pediococcus acidilactici* (AH5255). All strains of this group were homofermenter and grew well at 45°C. The strains AH3030**,** AH3031, AH3032 and AH3333 did not grow at 10°C or in the presence of 6.5% sodium chloride. They did not ferment D-arabinose, β-methyl-xyloside, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose and melezitose, but they did ferment ribose, galactose, D-glucose, D-fructose, N-acetyl-glucosamine, amygdalin, arbutin,

esculin, salicin and cellobiose. Furthermore, L-arabinose, D-xylose, D-mannose, mannitol, lactose, B-gentiobiose, maltose and saccharose were variously fermented. In addition, only the strain, *Lactococcus lactis* AH3533, was able to ferment starch and gluconate (Table 17).

Cluster F), this cluster contained four strains of *Lactobacillus* sp., BH1611, BH1711, BH181 and BH1911. They grew at pH 4.4, at 10 and 45°C and in the presence of 6.5% sodium chloride, but they did not grow at pH 9.6. They fermented ribose, xylose, hexoses, α-methyl-D-mannoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose and D-raffinose. They did not ferment melezitose, gluconate, inositol, mannitol, rhamnose, D-arabinose and β-methyl-xyloside (Table 18).

Table 17: Differencial carbohydrates fermentation by *lactococcus* and *Pediococcus* strains belonging to Cluster E.

	Lactococcus lactis AH3030 AH3031 AH3032	*Lactococcus lactis* AH3333	Lctococcus lactis ssp lactis AH3533	*Pediococcus pentsaceus* AH3233 AH3233 L AH3233 M AH3233 N AH3233 P	*Pediococcus pentosaceus* AH5655	*Pediococcus acidilactici* AH5255
L-Arabinose	+	-	-	-	+	+
D-Xylose	+	+	+	-	+	-
D-Mannose	+	+	+	-	+	+
Mannitol	+	+	+	-	-	-
Maltose	+	+	+	+	+	-
Lactose	+	+	+	-	-	-
Melibiose	-	-	-	-	-	-
Saccharose	+	+	+	-	-	-
Trehalose	+	+	+	+	+	-
Starch	w	w	+	w	-	-
B-Gentiobiose	+	+	+	+	+	w
D-Tagatose	-	-	-	+	+	+
Gluconate	w	w	+	w	w	-

+, Positive reaction; -, Negative reaction; w, Weak positive reaction

Table 18: Differential carbohydrates fermentation by *lactococcus* and *Pediococcus* strains belonging to Cluster F.

	Lactobacillus sp. strains			
	BH1611	BH1711	BH1811	BH1911
Erythritol	+	-	-	-
L-Xylose	-	+	+	+
Galactose	+	-	+	+
Dulcitol	-	+	-	+
α-Methyl-D-glucoside	-	+	-	+
Starch	+	+	-	+
D-Lyxose	-	+	-	-
L-Arabitol	+	-	-	-
2-Ceto-gluconate	+	+	+	-

+, Positive reaction; -, Negative reaction

Cluster I), contained strains of *Pediococcus pentosaceus and Lactobacillus plantarum.* Members of this cluster (seven strains of *Pediococcus pentosaceus* and one strain *Lactobacillus plantarum* BH1010) were homofermenters. All strains were able to grew at 10 and 45°C, at pH 4.4 and in the presence of 6.5% NaCl. However, D-arabinose, L-arabinose, β-methyl-xyloside, galactose, D-glucose, D-fructose, α-methyl-D-glucoside, N-acetyl glucosamine, amygdalin, arbutin, salicin, saccharose, xylitol and D-lyxose were fermented by members of this group. Adonitol was fermented by all strains except *Pediococcus pentosaceus* AH7777 and *Lactobacillus plantarum* BH1010. In addition, none of the strains fermented ribose, xylose, D-mannose, L-sorbose, rhamnose, dulcitol, sorbitol, α-methyl-D-mannoside, trehalose, D-raffinose, starch, gentiobiose, gluconate and D-arabitol. Melezitose, inositol, mannitol, maltose and lactose were fermented only by *Lactobacillus plantarum* BH1010.

6.3.4 *Minor clusters and stragglers (B, D, G and H).*

Cluster B contained one strain of *Lactobacillus fermentum;* cluster D contained two strains, *Lactobacillus fermentum* and *Lactobacillus* sp. One strain identified as *Lactobacillus plantarum* was assigned to cluster G. However, two other strains, *Lb. fermentum,* were grouped in cluster H. All strains were heterofermenters and grow at 45°C and in the presence of 6.5% of sodium chloride with the exception of strain BH2422. Members of cluster H were able to grow at pH 9.6 compared with strains of the other clusters. None of the strains fermented erythritol, adonitol, β-methyl-xyloside, D-lyxose, D-tagatose, D-fucose, L-arabitol and 2-keto-gluconate. However, strain BH2422 (cluster H) was also unable to ferment D-arabinose (Table 19). Table 20

highlights the different carbohydrates variously fermented by members of the clusters obtained at the similarity level of 79% (A-I).

Table 19: Differential carbohydrates fermentation by *lactobacillus* strains belonging to minor clusters.

	Cluster B	**Cluster D**		**Cluster G**	**Cluster H**	
	L.fermentum BH1511	*L. fermentum* BH0400	*Lactobacillus sp.* BH0500	*L. plantarum* BH0600	*L. fermentum* BH2722	*L. fermentum* BH2422
D-Xylose	-	+	+	-	+	-
L-Sorbose	-	-	+	-	-	-
Rhamnose	-	+	+	-	-	-
Mannitol	-	+	+	+	-	-
Sorbitol	-	+	+	-	-	-
α-Methyl-D-mannoside	-	-	+	+	-	-
Cellobiose	+	-	-	+	-	-
Lactose	+	+	-	+	+	+
Trehalose	-	-	-	+	-	-
Innulin	-	+	+	-	-	-
Melezitose	-	+	+	+	-	-
D-Raffinose	+	+	+	-	-	+
Starch	-	-	+	-	-	-
Xylitol	-	-	-	+	-	-
L-Fucose	-	+	+	-	-	-
D-Arabitol	-	-	+	+	-	-
Gluconate	-	-	+	-	+	+
5-Ceto-gluconate	+	+	+	-	-	-

+, Positive reaction; -, Negative reaction

Table 20: Phenotypic profiles of the 54 potent LAB isolates as determined by carbohydrate fermentation.

	Cluster A 18 isolates	Cluster B (BH1511)	Cluster C 6 isolates	Cluster D (BH0400 BH0500)	Cluster E 12 isolates	Cluster F 4 isolates	Cluster G (BH0600)	Cluster H (BH2722 BH2422)	Cluster I 8 isolates
D-Arabinose	5.5	0	0	0	0	0	0	50	100
L-Arabinose	55.5	100	33.33	100	41.66	0	0	100	100
Ribose	44.5	100	100	50	100	100	100	100	0
Adonitol	0	0	0	0	0	0	0	0	75
β-M-xyloside	0	0	0	0	0	0	0	0	100
D-Mannose	100	0	100	50	58.33	100	0	0	0
Rhamnose	0	100	50	0	0	0	0	50	0
Inositol	0	0	33.33	50	0	0	0	0	12.5
Mannitol	100	100	83.33	50	41.66	0	0	50	12.5
Sorbitol	50	100	83.33	0	0	100	0	50	0
Alpha-M-D-mannoside	38.88	0	50	50	0	100	0	50	0
β-M-xyloside	0	0	16.2	50	0	50	0	0	100
Cellobiose	100	0	100	50	100	100	0	50	25
Maltose	100	100	100	50	91.66	100	100	100	12.5
Lactose	10	100	100	100	41.66	100	100	100	12.5
Melibiose	100	100	66.7	50	0	100	100	100	87.5
Saccharose	100	100	100	100	41.66	100	100	100	100
Trehalose	100	0	100	50	91.66	100	0	0	0
Melezitose	100	100	83.33	50	0	0	0	50	12.5
D-Raffinose	94.5	100	50	0	0	100	100	100	0
Starch	0	0	0	0	8.33	75	0	50	0
Gentiobiose	100	100	100	50	91.66	0	0	100	0

Numbers indicate the percent positive reaction of each test for the isolates of each cluster.

6.3.5 *Genotypic characterization of selected LAB strains*

Sequences of the 16S rRNA gene (approximately 1,500 bp) of ten LAB isolates, BH0900, BH1511, AH3133, BH0500, AH5655, AH3030, BH1711, BH0600, BH2422, and AH3433A, were determined. The 16S rRNA nucleotide sequences of the isolates were aligned with homologous regions from various LAB, and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 14). The BLAST analysis of 16S rRNA gene sequences of the selected strains showed alignments of these sequences with reported 16S rRNA genes in the gene bank. The nucleotide sequences were deposited in GenBank, and accession numbers for strains BH0900, BH1511, AH3133, BH0500, AH5655, AH3030, BH1711, BH0600, BH2422 and AH3433A were obtained (KF178303, KF178304, KF178305, KF178307, KF178308, KF178306, KF178310, KF178311, KF178312 and KF178309, respectively). However, the 10 isolates were assigned to seven species, *Lactobacillus plantarum*, *Lb. fermentum*, *Lactococcus lactis*, *Lb. ingluviei*, *Pediococcus pentosaceus*, *Lb. acidipiscis* and *Weissella cibaria*.

On the basis of phylogenetic data obtained, strain BH0900 showed similarity (99%) with *Lactobacillus plantarum* WCFS1 (075041.1) and *Lactobacillus pentosus* 124-2 (029133.1) as well as with *Lactobacillus paraplantarum* DSM 10667 (025447.1) and *Lactobacillus plantarum* NRRL B-14768 (042394.1). However, strain BH1511 shares 99% similarity with *Lactobacillus fermentum* IFO 3956 (075033.1) and 96% similarity with *Lactobacillus gastricus* Kx156A7 (029084.1). On the other hand, strains AH3133 and AH3030 share 99% similarity with *Lactococcus lactis* subsp. *lactis* NCDO 604 (040955.1), *Lactococcus lactis* subsp. *hordniae* NCDO 2181 (040956.1), *Lactococcus lactis* subsp. *cremoris* SK11 (074949.1), *Lactococcus lactis* subsp. *cremoris* NCDO 607 (040954.1) and 92% similarity with *Lactococcus plantarum* DSM 20686 (044358.1). Furthermore, 99% similarity was shared between isolate BH0500 and *Lactobacillus ingluviei* KR3 (028810.1). In addition, two strains, AH5655 and AH3433A, were closely related (99%) to *Pediococcus pentosaceus* DSM 20336 (042058.1) and showed more than 98% similarity to *Pediococcus stilesii* LMG 23082 (042401.1). One strain, BH1711, displayed 98% similarity with *Lactobacillus acidipiscis* FS60-1 (024718.1). Strain BH0600, which was phenotypically identified as *Lactobacillus plantarum*, was genotypically related (99%) to *Lactobacillus ingluviei* KR3 (028810.1). Finally, strain BH2422 was considered *Lactobacillus fermentum* based on phenotypic characterization; however, 16S rRNA gene sequence analysis revealed that this strain was related to *Weissella* and shared 99% sequence similarity with *Weissella cibaria* II-I-59 (036924.1).

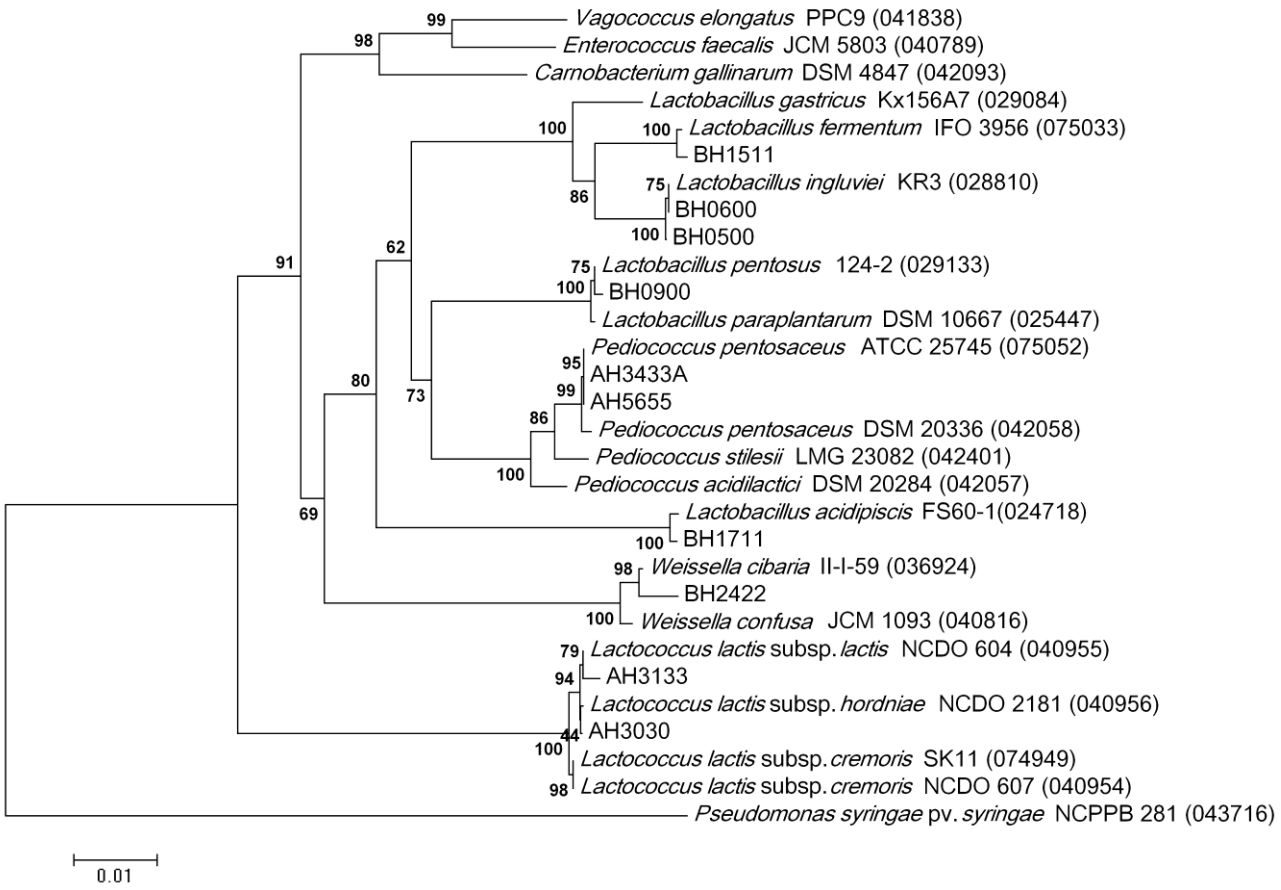


Figure 14: Phylogenetic tree based on 16S rDNA sequence analysis, showing the phylogenetic placement of selected LAB strains isolated from pollen grains. The tree was constructed by the neighbor-joining method, and *Pseudomonas syringae* pv. *syringae* was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

6.4 DISCUSSION

The effectiveness of LAB is a strain-dependant aspect. This trait may be obtained by genetic manipulation or, as is frequently the case, searching for new desirable strains in natural niches. Plants, foods, fermented products, animals and humans constitute natural ecological systems and good sources for LAB. To the best of our knowledge, antagonism of LAB against human bacterial gastrointestinal pathogens is the most important feature for selecting such a strain designed for the human gut.

Here, we described isolation of LAB from raw pollen grains showing antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria. The 54 LAB effective strains were characterized by means of phenotypic tests. The relationships among the phenotypically characterized strains of LAB were determined by cluster analysis. It is well known that pollen grains are sterile before anther opening. Flowers and their constituents are parts of the plant phylloplane. Stirling and Whittenburg (1963) suggested that the LAB are not usually part of the normal microflora of the growing plant and indicated the role of insects in the spread of these organisms. LAB commonly found on fresh herbage and in silage have been investigated (Weise, 1973; Woolford, 1975). Nilsson and Nilsson (1956) found that the predominant LAB during silage fermentation were streptococci and lactobacilli, with *Lactobacillus plantarum* the species most frequently recovered. Other studies (Mundt and Hammer, 1968; Mundt *et al*., 1969) reported the occurrence of pediococci and lactobacilli on leaving or decayed plants. Lactobacilli commonly share the habitat phyllosphere with species of the genera *Leuconostoc*, *Pediococcus* and *Weissella*. Species frequently recovered from the leaves include *Lb. plantarum*, *Lb. paracasei*, *Lb. fermentum*, *Lb. brevis* and *Lb. buchneri* (Hammes and Hertel, 2006), which is in line with our results. Part of the accumulated information about the occurrence of lactobacilli (and other LAB members) on plants is derived from microbiological studies of the fermentation process. Thus, the microbial population upon initiation of the process is known for several plants (grasses, cabbage, silage raw materials, carrots and beets, olives and fruits such as grapes and pears, etc.). But scarce information about the occurrence of LAB on flowers and pollens is available in the literature. Indeed, we report in this work the occurrence of LAB in pollen grains and for the first time the isolation and characterization of several species belonging to LAB. The results reported here indicate that 54 isolates (25%) of the total antagonistic isolates (216 strains) inhibit indicator bacteria. Inhibition caused by hydrogen peroxide and organic acids was ruled out, as the producer strains were cultured anaerobically and the culture supernatant was neutralized (pH 6.5) before assaying the antimicrobial activity. This study indicates that the compound inhibiting the microbial growth in the neutralized cell-free supernatant was not organic acid or hydrogen peroxide commonly produced by many LAB (Helander *et al.*, 1997).

As reported by Daeschel *et al.* (1987), certain LAB protect plants by producing antagonistic compounds (Visser *et al.*, 1986) contributing to inhibition of the plant pathogens *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae*.

Furthermore, LAB are well known for their antagonism towards other Gram-positive bacteria, especially taxonomically related species (*Listeria* spp., *Bacillus* spp. *Micrococcus* spp., etc.), and Gram-negative plant and animal pathogens, such as *Escherichia coli*, *Salmonella* spp., *Helicobacter pylori* and *Pseudomonas aeruginosa*. Contrary to what is believed, that LAB are more potent in inhibiting Gram-positive bacteria than Gram-negatives bacteria, which is claimed for the type of cell wall of the target microorganism, this study reveals that Gram-negative bacteria used in this study (*E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Shigella* sp.) were susceptible to cell-free supernatants from tested LAB, especially the strains 9 (*Pediococcus pentosaceus* AH3433E), 12 (*Lactobacillus plantarum* BH1010), 14 (*Lactobacillus plantarum* BH0600), 16 (*Lactobacillus* sp. BH1811), 19 (*Pediococcus acidilactici* AH5255), 29 (*Lactobacillus plantarum* BH0800), 39 (*Lactobacillus plantarum* BH1411), 41 (*Lactobacillus plantarum* BH0104) and 47 (*Lactobacillus plantarum* BH0102). These results are in accordance with earlier results reported by Trias *et al.* (2008), who showed that most LAB originating from fruits and vegetables displayed good antagonistic activity against foodborne pathogens, such as, *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli*. Several indicator strains displayed different degrees of susceptibility towards antimicrobial compounds from a given producer strain; for example, strain 46 (*Lactobacillus* sp. BH2122) inhibits strongly *E. coli*, *Salmonella typhimurium* and moderately inhibits *Pseudomonas aeruginosa* but is inactive towards *Shigella* sp. The indicator strain *L. innocua* was used in this study instead of *L. monocytogenes*, as the two microorganisms show similar physiological properties with the difference that the former does not belong to the pathogenic species of *Listeria*. Moreover, some papers have reported a greater sensitivity of *L. monocytogenes* towards some antibacterial compounds than *L. innocua* (Con *et al.*, 2001; Mataragas *et al.*, 2003).

It is well known that the presence of lactobacilli is important for maintenance of the intestinal microbial ecosystem (Sandine, 1979). They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes* (Ashenafi, 1991; Harris *et al.*, 1992), *Escherichia coli* and *Salmonella* spp. (Château *et al.*, 1993; Drago *et al.* 1997). This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, and bacteriocins. Our results agreed with the latter statements; therefore, our isolates are strong candidates for clinical use during gut treatment regimes. Furthermore, a major advantage of using LAB as biocontrol agents is that they are considered GRAS (generally recognized as safe) and usually comply with all recommendations for food and drug products (Stiles and Holzapfel, 1997). Moreover, LAB are natural colonizers of fresh plant products and have been previously described as good antagonists of several bacteria and fungi (Batish *et al.*, 1997; Sathe *et al.*, 2007).

Siezen et *al.* (2010) hypothesized that the fermentative profile reflects the original habitat and that lactose utilization is less prevalent in plant isolates with respect to those from cheese and the human gastrointestinal tract. Indeed, lactose was fermented by most

isolates in the present study, except isolates of *Pediococcus* from clusters I and E and isolates of *Lactobacillus* sp. from cluster A. The inability of plant-related LAB to ferment lactose was presumably due to the relatively recent acquisitions, via horizontal gene transfer and subsequent natural selection, of lactose metabolic genes, which are often plasmid encoded in dairy and human strains (Siezen *et al.*, 2005). Contrary to the findings of Cagno et *al.* (2010), who studied *Lactobacillus plantarum* from vegetables and fruits, all *Lactobacillus plantarum* isolates of this study used this carbon source.

Overall, all isolates fermented maltose and cellobiose except *Pediococcus acidilactici* AH5255 (cluster E) and *Pediococcus pentosaceus* AH3433, AH3433A, AH3433B, AH3433C, AH3433D and AH3433E (cluster I). Strain AH7777 (*Pediococcus pentosaceus*; cluster I*)* fermented cellobiose but not maltose. Also, cellobiose was not fermented by *Lactobacillus fermentum* BH0400, *Lactobacillus* sp. BH0500 (cluster D), members of cluster H (*Lactobacillus fermentum* BH2722, and BH2422) and members of clusters B and G. Arabinose, glucose, fructose, mannose, mannitol, B-gentiobiose, melezitose, melibiose, saccharose and trehalose were variously fermented. These carbon sources correspond mainly to those prevalent in the plant kingdom (Buckenhüskes, 1997). Similar phenotypic profiles were found for *Lactobacillus plantarum* isolated from Thai fermented fruits and vegetables (Buckenhüskes, 1997; Siezen *et al.*, 2005; Tanganurat *et al.*, 2009). Glycosides such as amygdalin were not used only by isolates of clusters H, D, B and C, salicin was not used only by eight isolates belonging to cluster A, (*Lactobacillus* sp. BH0700, 0701, 0702, 0703, 0704, 0705, 0706 and 0707). On the other hand, arbutin was used by isolates of all clusters. These glycosides are typically found in vegetables.

Starch is a reserve polysaccharide in pollen grains, and starch-hydrolyzing strains belong to the clusters E, F, and H. According to the fermentation profile, it seems that the isolates assimilate variously a panel of carbohydrates that reflects their enzymatic and genetic potentials. Furthermore, these traits were shared with LAB of dairy or animal origin. Based on the limited number of tests used, phenotypic profiles did not cluster the isolates according to the original habitat. This was probably because the studied isolates were obtained from raw pollen samples having a very similar chemical composition. It could also have been because the pollen residing LAB were of animal as well as plant origin. Nevertheless, phenotypic profiling was useful to understand the manifestations of environmental adaptation, which will be reflected in the technological processes.

From the different phenotypic clusters, ten selected isolates (displaying a good assimilation activity) were identified by means of 16S rRNA gene sequencing. It is well known that the species *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes. In the present results, misidentification of some isolates to defined species was encountered. More molecular techniques should be used for determination of the taxonomic status of these strains. The occurrence of *Weissella cibaria, Lactobacillus ingluviei* and *Lactobacillus acidipiscis* in pollen grains is in accordance with the

ubiquity of these bacteria in nature (Dworkin *et al.*, 2006). In fact, insects, honeybees, soil, water and animal and human feces are main microbial contamination sources of pollens (Belhadj *et al.*, 2010; 2012). From another point of view, misidentification of some isolates by phenotypic traits is probably due in part to the limited characters used for this purpose, or by the similarity of the metabolic patterns expressed by the isolates, even if they belong to different genotypic ranks. As reported elsewhere (Rodas *et al.*, 2003), physiological and biochemical criteria used for LAB strain identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, a clear identification to species level by simple phenotypic tests may be troublesome and inaccurate. Molecular methods used for discrimination of LAB strains to genus and species level are more efficient than phenotypic approaches.

A preliminary study on LAB associated with pollens having remarkable antimicrobial activity is reported here. The uses of these strains for biocontrol or bio-preservation purposes should be evaluated. Phenotypic traits do not reveal the real taxonomic position of some isolates; therefore, exploitation of other molecular methods for exact identification of these bacteria is of great scientific and practical interest. In addition, the search for new LAB exhibiting a wider spectrum of antimicrobial activities from pollen grains that can be used in human health, agriculture and the food industry is of great importance. Furthermore, studies on other biological and biotechnological criteria of these isolates as well as their safety aspects are necessary. Finally, it seems that pollens and possibly other beehive products are predominant sources for isolation of LAB with potent applications.

Chapter 07

Probiotic

Lactobacilli

7. Some Probiotic Traits of *Lactobacillus* Strains Isolated From Pollen

7.1 INTRODUCTION

The historic use of bacteria that produce lactic acid and collectively are designated lactic acid bacteria (LAB) is well documented for a variety of food fermentations, some even dating back to the earliest written records. However, less exposed is the impact of LAB in the diet of our far-away ancestors that lived over a million of years ago? There is considerable support for the hypothesis that lactobacilli and other notably plant-related LAB have been consumed in large amounts in neolithic times (De Vos, 2011). This so called paleo-diet may have contained over a million more microbes than our present foods, resulting in a high and continuous load of LAB. In retrospect, this provides an explanation why some LAB have developed intimate interactions with our body and several LAB are successfully marketed as probiotics (Saxelin *et al.*, 2005; GPM, 2010). Traditionally LAB have been considered to include low G+C content Gram-positive bacteria included in the phylum Firmicutes that are used as starters for industrial food fermentations, notably those based on raw materials derived from milk, meat and plants. These fermentations together with probiotic products represent a total global market value of over 100 Billion Euro (GPM, 2010; OECD-FAO, 2010). Economically by far the most important products derive from industrial dairy fermentations and include cheese, yoghurt and other fresh dairy produce. These fermentations are initiated by well-known genera of LAB that include *Lactobacillus*, *Lactococcus* and *Streptococcus*. However, the market for probiotic bacteria in foods and supplements is the most rapidly growing segment in the fast moving consumer goods and expected to grow by 10% each year (GPM, 2010). Most of the applied probiotic bacteria are *Lactobacillus* spp. However, approximately one third are Bifidobacteria, a group of bacteria with a high G+C content included in the phylum Actinobacteria that also produce lactic acid but always in combination with acetic acid. Bifidobacteria are almost exclusively found in association with animal hosts (Vaughan *et al.*, 2005). Hence, it is no surprise that some *Bifidobacterium* strains are also marketed as probiotic bacteria (Saxelin *et al.*, 2005; GPM, 2010). Increased involvement in nosocomial infections and readily transfer antibiotic resistance, concerns about the safety of using enterococcal probiotics have been expressed (Franz *et al*., 1999; Lund and Edlund, 2001). Other less common probiotic strains are *Escherichia coli*, *Bacillus* and *Saccharomyces* species. In Central Europe, a strain of *E. coli* has been used for treatment of various intestinal diseases. One character of importance for protection against enteropathogens is competition for binding sites, which has been demonstrated in pigs (Castagliuolo et al., 1996). *Bacillus subtilis* is an aerobic endospore-forming saprophyte widely distributed in the natural environment. The probiotic activity of *Bacillus* spores is still unclear. Furthermore, biochemical, physiological and phylogenetic analyses of commercial products have showed that several products are mislabelled and contain species with high levels of drug resistance (Hoa *et al*., 2000). *Saccharomyces*, yeast that is used in the production of beers and wines. Antibiotics do not affect the yeast, which could be an advantage in probiotic preparations used

for preventing disruptions in the normal microflora by antimicrobial agents. *Saccharomyces boulardii* has been shown to produce a protease interfering with *Clostridium difficile* toxin A in rats (Sullivan and Nord, 2002). LAB have been classified as 'generally recognized as safe' (GRAS) due to their general occurrence in many fermented and non-fermented food products and also being part of the human commensal micro-flora. There have however been a few reported cases on clinical infections such as endocarditis, bacteraemia, and urinary tract infections caused by these microbial species, though in all these cases, patients had underlying conditions which predisposed them to infections particularly in the case of endocarditis. *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Leuconostoc* species and *Lactobacillus casei* (*paracasei*) have been cited in some non-enterococcal LAB endocarditis cases. In view of this, it is relevant to have a more thorough safety assessment of LAB before their uses as live cultures for varying applications in the food and feed industry (Adimpong *et al*., 2012). It is well established that probiotic traits are strain dependent aspects. However, when the probiotic microorganism is being to be delivered for the gut, tolerance to stomach and intestinal harsh conditions is needed. Over the past few decades, probiotic LAB have been very intensively investigated. Most of the LAB isolated from raw plants and plant products constitute an important source of new functional bacteria that can play roles in GI of humans and animals or food processing (Leroy and De Vuyst, 2004).

In searching for interesting strains with probiotic potential, studies on natural plant derived products as a source of new isolates are rapidly accumulating. The objective of this study was to characterize and establish the functional characteristics of potential probiotic *Lactobacillus* strains isolated from bee pollen grains. The isolates were preliminarily selected on the basis of acid and bile tolerance, and the selected isolates were further screened for various functional properties such as antimicrobial activity against pathogenic bacteria, and hydrophobicity ability. In addition, the probiotic candidate isolates were characterized by means of phenotypic and genotypic (16S rRNA gene sequencing).

7.2 MATERIALS AND METHODS

7.2.1 *Isolation of Lactobacillus strains*

During flowering seasons, honey bee foragers are kinched, by a sterile stainless steel or plastic forceps. The pollen pellets were then recovered using humidified sterile cotton swabs by gently touching the pollen pellets attached to the posterior legs of the bee. The swabs were transported to the laboratory at low temperature (+5 °C) and analyzed or further maintained at refrigeration until use. Each swab was then introduced in a screw capped glass tube containing 10 mL LBS broth (pH 6.5) (Rogosa et al., 1953), and incubated anaerobically at 37°C for at least two days. Afterwards, from each tube, serial dilutions were prepared in peptone water (0,1% peptone and 0,1% of Tween 80), and from the appropriate dilution, aliquots (100 µL) were spread cultured on LBS agar. Plates were incubated anaerobicaly (BBL GasPack System) at 30°C for 72 h. Strains were stored in MRS broth (Fluka) supplemented with glycerol (15% v/v final concentration) at -20 °C. They were activated twice before each experiment in MRS broth containing 0.05 % L-cysteine hydrochloride at 37°C for 48 h.

7.2.2 *Antimicrobial activity assay*

Overnight culture of the each strain was used to inoculate (1% v/v) 10 ml of MRS broth. After 18 h of incubation at 37°C, cells were removed by centrifugation (4500 g for 15 min). The supernatant was filter-sterilized (0.22 µm pore size; Serva, Heidelberg, Germany) to eliminate the possible presence of viable cells. pH of the supernatant was neutralized to 6.5 with 1 N of NaOH. Then they were stored at -18°C until use.

To determine the antimicrobial activity of the *Lactobacillus* strains, agar well diffusion method was used as follows. 100 µL of standardized indicator strain inoculums [A_{600}= 0.5] (*Escherichia coli and Staphylococcus aureus*) were mixed with 9.0 ml Muller-Hinton (MH) soft agar (0.7% agar, w/v), well vortexed and poured into pre-prepared plates containing 18 ml of MH agar. Wells (6 mm in diameter) were cut with a sterile cork borer and filled with 70 µL of neutralized supernatant culture. The plates were left for 1 h at room temperature before incubating them for 24 h at 37°C. After that inhibition zones were recorded.

7.2.3 *Tolerance to simulated gastric juice*

The acid tolerance of studied *Lactobacillus* strains was studied in simulated gastric juices as described by Charteris *et al*. (1998). The simulated gastric juices prepared by PBS buffer solution with pepsin (0.3%, w/ v). The buffer solutions were prepared by adjusting the pH to 1.5, 2.0, 3.0 and 6.2 (control) with 6 N HCl, and filter sterilized (0.45 µm pore size). After thorough mixing, 10 mL of each pH solution was taken in sterilized test tubes. Each cell suspension of the selected *lactobacillus* cultures containing about 10^9 CFU/mL was added to pH solution of 1.5,

2.0, 3.0 or control (6.4) and well mixed. After 3 h of incubation, 1 mL from each pH solution was serially diluted with 0.85% sterile saline. Appropriate dilutions were spread-plated onto MRS agar (containing 0.05% L-cystein hydrochloride) and incubated under anaerobic conditions at 37 °C for 72 h. The colony forming units were then estimated. The assay was performed in triplicate.

7.2.4 *Bile salt tolerance*

The ability of the isolates to grow in the presence of bile was determined using the method of Vinderola and Reinheimer (2003). The bile salt solutions were prepared using bile powder (oxgall, Sigma), at the final concentrations of 0.3%, 0.5%, and 1%. MRS broth without oxgall (pH 6.2) was used as the control. All the solutions were autoclaved, and 10 mL of each solution was transferred into sterile test tubes. Cell suspensions containing about 10^9 CFU/mL were added to each solution, i.e., 0.3%, 0.5%, 1% and control, and incubated at 37°C under anaerobic conditions. After 10 h incubation, 1 mL of each culture was diluted in sterile 9 mL of 0.85% saline solution, and the appropriate dilutions were spread plated on MRS agar. Plates were incubated under anaerobic conditions at 37°C for 72 h and the colony forming units estimated. Survival rate was calculated according to the following equation: Survival rate (%) = [Log CFU N_1/ Log CFU N_0] x 100. N_0 and N_1 were number of counted bacteria at time 0 and 10 hours respectively. The assay was performed in triplicate.

7.2.5 *Antibiotic susceptibility*

For these *Lactobacillus* isolates, the MICs of 10 antibiotics and 2 proton pump inhibitors were determined using the broth microdilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000). Briefly, colonies from solid media were used to make an even suspension (equivalent to the 0.5 McFarland turbidity standard) in Mueller–Hinton broth (Fluka) containing 1% glucose. Ten-microlitre aliquots of this suspension were inoculated into each well of the 96 bottomed wells plate containing each 180 µL Mueller–Hinton broth (supplemented with 1% glucose). The drugs analyzed included amoxicillin, colistine, omeprazol, oxacilline, clindamycin, chloramphenicol, ciprofloxacine, roxithromycin, oxytetracycline, penicillin G, metronidazole, and ranitidine. Following 24-h incubation at 37°C under anaerobic conditions, MIC values were determined. Results were interpreted according to the cut-off levels proposed by (Charteris *et al*., 1998; EFSA, 2005; 2008; 2012].

7.2.6 *Cell surface hydrophobicity*

The *in vitro* cell surface hydrophobicity was determined by the bacterial adherence to hydrocarbon assay modified from the methods of Rosenberg *et al*. (1980). Briefly, the test bacteria were grown in MRS broth at 37 °C under anaerobic conditions. The 18–24 h (stationary phase) test culture was harvested after centrifugation at 12 000 *g* for 10 min at 5 °C, washed twice and resuspended in 50

mM K_2HPO_4 buffer (pH 6.5) to an optical density ($OD_{560\ nm}$) of 0.8-1.0 (A_0) measured spectrophotometrically. A portion of 0.6 mL of n-hexadecane was added to 3 mL of bacterial suspension. The mixture was blended using a vortex mixer for 120 sec. The tubes were allowed to stand at 37°C for 30 min to separate the two phases. The aqueous phase was carefully removed and the $OD_{560\ nm}$ of the aqueous phase (A) was measured. Hydrophobicity was calculated from tow replicates as the percentage decrease in the optical density of the initial aqueous bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (H %) of the strain adhering to hexadecane was calculated using the equation:

H (%) = $[(A_0 – A)/A_0) \times 100]$.

7.2.7 *Phenotypic identification of Lactobacillus strains*

All isolates were tested for Gram stain and catalase production (Norris *et al.*, 1981). Gram-positive, catalase-negative isolates were checked for gas (CO_2) production from glucose in MRS broth containing inverted Durham tubes (Müller, 1990), growth at 15 and 45°C, growth in the presence of 6.5 and 18% (w/v) of NaCl, and growth in MRS broth with pH 4.2 and 9.6 (Shaw and Harding, 1984; Schillinger and Lũcke, 1987). Further biochemical characterization was carried out using API 50 CH galleries (API System, BioMerieux, Montalieu Vercie, France) following the manufacturer's recommendations. The APILAB Plus computer-aided identification program version 4.0 (BioMérieux) was used to analyze the carbohydrate fermentation profiles obtained with the identification strips.

7.2.8 *Genotypic identification of Lactobacillus strains*

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using two universal primers: forward primer, 5' AGAGTTTGA TCCTGCTCAG-3', and reverse primer, 5'-AAGGAGGTGA TCCAAGCC-3', designed from base positions 8 to 27 and 1541 to 1525, respectively. These are the conserved zones within the rRNA operon of *Escherichia coli* (Gurtler and Stanisich, 1996). Genomic DNA of each strain was used as a template for PCR amplification (35 cycle, 94°C for 30 sec denaturation, 65°C for 1 min primer annealing and 72°C for 2 min extention). The amplified ~1.5 kb PCR products were cloned in the pEH-T Easy vector (Promega, Madison, WI, USA). The *Escherichia coli* DH5α (F^- *supE44 Φ80 δlacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17* (r_k^-, m_k^+) *deoR thi-1* λ^- *gyrA96 relA1*) (Invitrogen Life Technologies) was used as a host strain. All recombinant clones of *E. coli* were grown in LB broth media with the addition of ampicillin, IPTG, and X-gal for screening. DNA electrophoresis, DNA purification, restriction, ligation, and transformation were all performed according to the methods previously described by Sambrook *et al*. (1989).

7.2.9 *DNA sequencing and phylogenetic analyses*

The nucleotide sequence of 16S rRNA genes was determined on both strands using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the automated DNA sequencer ABI Prism® 3100-Avant Genetic Analyser (Applied Biosystems). The 16S rDNA sequences analysis was performed by means of the BLAST program (www.ncbi.nlm.nih.gov/blast). Phylogenetic and molecular evolutionary analyses were conducted using the molecular evolutionary genetics analysis (MEGA) software version 4.1. Distances and clustering were calculated using the neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1000 resamplings (Felsenstein, 1985). The sequences were deposited in the GenBank database using the web-based data submission tool, Sequin (http://www.ncbi.nlm.nih.gov/Sequin,).

7.3 RESULTS

7.3.1 *Antimicrobial activity*

Results of the antimicrobial activity are presented in Table 21 and Figure 15. Except the tow isolates (BH1511 and BH1525), the studied lactobacilli were active against Gram-positive and Gram-negative bacteria. Neutralized supernatants of the culture strongly inhibited the growth of *E. coli* more than *Staphylococcus aureus*, especially those obtained from the isolates BH1541, BH1507, and BH1491 (inhibition zone from 31 to 35 mm). The other five supernatants exhibit similar inhibitory effect as determined by inhibition zone diameter (20-22 mm). Weak activity was observed with the isolates BH1511, BH1525, and BH1480 against *Staphylococcus aureus*. Tow strains (BH1507 and BH1491) display good antagonism towards the Gram-positive bacterium with inhibition diameters of 29 and 31 mm respectively. In general, all strains display less or more antagonism against tow different pathogenic bacteria.

7.3.2 *Characterization*

The carbohydrate fermentation patterns of the strains were obtained in our laboratory using the API 50 CHL kits. Analysis of fermentation patterns identified the organisms as *Lactobacillus plantarum* (three strains), *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus pentosus*, *Lactobacillus* sp. (tow strains), *Lactobacillus lactis* and *Lactobacillus sakei*. The identification of the newly isolated lactobacilli was based on both phenotypic and molecular methods. Genus level ascribing of the isolates was performed according to Axelsson (2004) and the methods described in Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986) and the prokaryotes (Dworkin *et al*., 2006).

Table 21: Antimicrobial activity of *Lactobacillus* strains isolated from pollen grains against *Escherichia coli* and *Staphylococcus aureus*.

Strains (Phenotype)	*Escherichia coli*	*Staphylococcus aureus*
Lactobacillus plantarum BH1541	**31**	**24**
Lactobacillus acidophilus BH1495	**22**	**24**
Lactobacillus plantarum BH1507	**33**	**29**
Lactobacillus fermentum BH1509	**22**	**23**
Lactobacillus pentosus BH1511	**N**	**13**
Lactobacillus sp. BH1398	**22**	**16**
Lactobacillus lactis BH1525	**N**	**11**
Lactobacillus sp. BH1480	**21**	**13**
Lactobacillus plantarum BH1491	**35**	**31**
Lactobacillus sakei BH1503	**20**	**18**

Values represent inhibition zone diameter (mm).
N, null

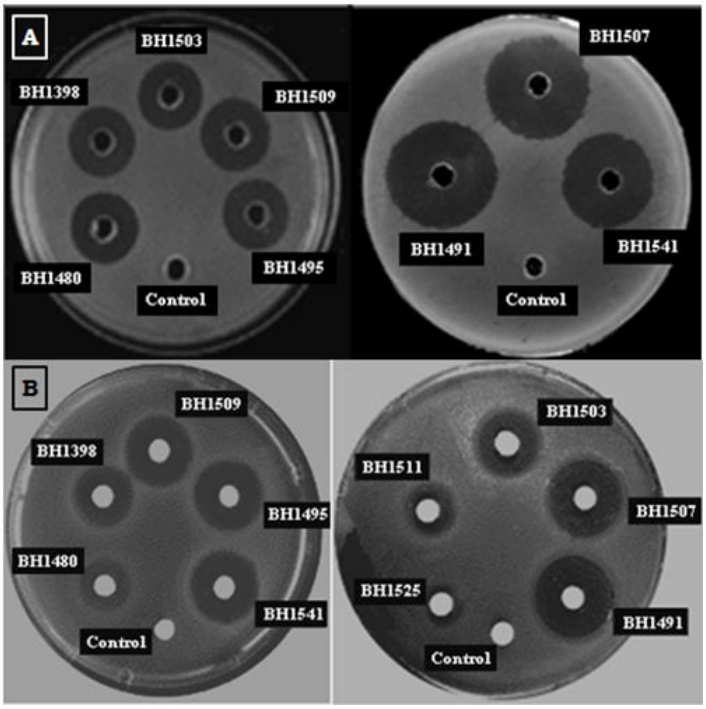


Figure 15: Well diffusion plate showing growth inhibition of *Escherichia coli* (A), and *Staphylococcus aureus* (B) by ten isolated *Lactobacillus* species from pollen.

The data obtained showed that the isolated strains appear in a bacillus form, gram-positive, catalase negative, and non-motile. The carbohydrate utilization profiles of the isolates were also investigated using API 50 CH strips (Table 22). The results indicate that all strains ferment maltose, saccharose, trehalose, melibiose, raffinose, D-turanose, D-arabitol, L-arabinose, galactose, fructose, sorbitol, N-acetyl glucosamine, and arbutine, but could not ferment glycogene, inuline, D-tagatose, D-fucose, L-fucose, xylitol, 2-keto gluconate, erythritol, adonitol, Beta-methyl xyloside, dulcitol, inositol, and D-methyl-D-glucoside. However, gentiobiose was not fermented only by the strains BH1495 and BH1509. Lactose, gluconate, ribose, and mannitol were not used only by the strain BH1398. The strain BH1509 was a starch degrading bacterium. Table 22 indicates results of charbohydrate fermentation that differenciate between the newly isolated lactobacilli.

Sequences of the 16S rRNA gene (approximately 1,500 bp) of the *Lactobacillus* isolates; BH1541, BH1495, BH1507, BH1509, BH1511, BH1398, BH1525, BH1480, BH1491, and BH1503 were determined. The taxonomic position of all 10 genotypes was determined by sequence analysis of about 1,500 bp of the 5´ region of the 16S rDNA gene. The 16S rRNA nucleotide sequences of the isolates were aligned with homologous regions from various Lactobacilli, and the phylogenetic tree was constructed by neighbor-joining method Fig. 16. The BLAST analysis of 16S rRNA gene sequences of the selected strains showed alignments of these sequences with reported 16S rRNA genes in the gene bank. The nucleotide sequence data was deposited to the gene bank and gene bank accession numbers for the strains BH1541, BH1507, BH1491, BH1509, BH1398, BH1480, BH1495, BH1511, BH1503, and BH1525 were obtained (KC845564, KC857459, KC857460, KC857461, KC857462, KC857463, KC857464, KC857465, KC857466, and KC857467 respectively). On the basis of phylogenetic data obtained the strains BH1509 showed similarity (99%) with *Lactobacillus fermentum* IFO 3956 (075033.1) and shares 95% similarity with *Lactobacillus gastricus* strain Kx156A7 (029084.1) as well as with *Lactobacillus mucosae* strain CCUG 43179 (024994.1), *Lactobacillus equigenerosi* strain NRIC 0697 (041566.1) and *Lactobacillus ingluviei* strain KR3 (028810.1). However, the strain BH1507 shares 99 % similarity with *Lactobacillus plantarum* WCFS1 (075041.1), *Lactobacillus pentosus* 124-2 (029133.1), *Lactobacillus plantarum* NRRL B-14768 (042394.1), *Lactobacillus paraplantarum* strain DSM 10667 (025447.1), *Lactobacillus plantarum* subsp. *argentoratensis* DK0 22 (042254.1), and *Lactobacillus fabifermentans* LMG 24284 (042676.1). Whereas, strain BH1491 shares 99 % of similarity with *Lactobacillus plantarum* WCFS1 (075041.1), *Lactobacillus pentosus* 124-2 (029133.1), *Lactobacillus paraplantarum* strain DSM 10667 (025447.1), *Lactobacillus plantarum* subsp. *argentoratensis* DK0 22 (042254.1), and *Lactobacillus fabifermentans* LMG 24284 (042676.1).

Table 22: Carbohydrates fermentation profile of lactobacilli isolated from pollen grains.

	***Lactobacillus* strains**									
	BH1541	BH1495	BH1507	BH1509	BH1511	BH1398	BH1525	BH1480	BH1491	BH1503
Carbohydrate :										
Aesculine	+	-	+	-	+	+	+	+	+	+
Salicine	+	+	+	+	+	-	+	+	+	-
Cellobiose	+	-	+	-	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+	+	+	-
Gentiobiose	+	-	+	-	+	+	+	+	+	+
Melezitose	+	+	+	+	-	+	-	-	+	+
Starch	-	-	-	+	-	-	-	-	-	-
D-Lyxose	-	-	-	-	+	-	+	-	-	-
L-Arabitol	-	-	-	+	-	-	-	-	-	-
Gluconate	+	+	+	+	+	-	+	+	+	+
5-keto gluconate	-	+	-	+	-	-	-	-	-	+
Glycérol	-	+	-	+	+	+	+	+	-	+
D-Arabinose	-	-	-	+	-	-	-	-	-	-
Ribose	+	+	+	+	+	-	+	+	+	+
D-Xylose	-	+	-	+	+	-	+	+	-	+
L-Xylose	-	-	-	+	-	-	-	-	-	-
Glucose	+	-	+	+	+	+	+	+	+	-
Mannose	+	-	+	-	+	+	+	+	+	-
L-Sorbose	-	-	-	+	-	-	-	-	-	-
Rhamnose	-	+	-	+	-	-	-	-	-	+
Mannitol	+	+	+	+	+	-	+	+	+	+
L-Methyl-D-mannoside	+	-	+	+	-	-	-	-	+	-
Amygdaline	+	-	+	-	+	+	+	+	+	-

All strains ferment Maltose, Saccharose, Trehalose, Melibiose, Raffinose, D-Turanose, D-Arabitol, L-Arabinose, Galactose, Fructose, Sorbitol, N-Acetyl glucosamine, and Arbutine, but they did not ferment Glycogene, Inuline, D-Tagatose, D-Fucose, L-Fucose, Xylitol, 2-keto gluconate, Erythritol, Adonitol, Beta-Methyl xyloside, Dulcitol, Inositol, and D-Methyl-D-glucoside.

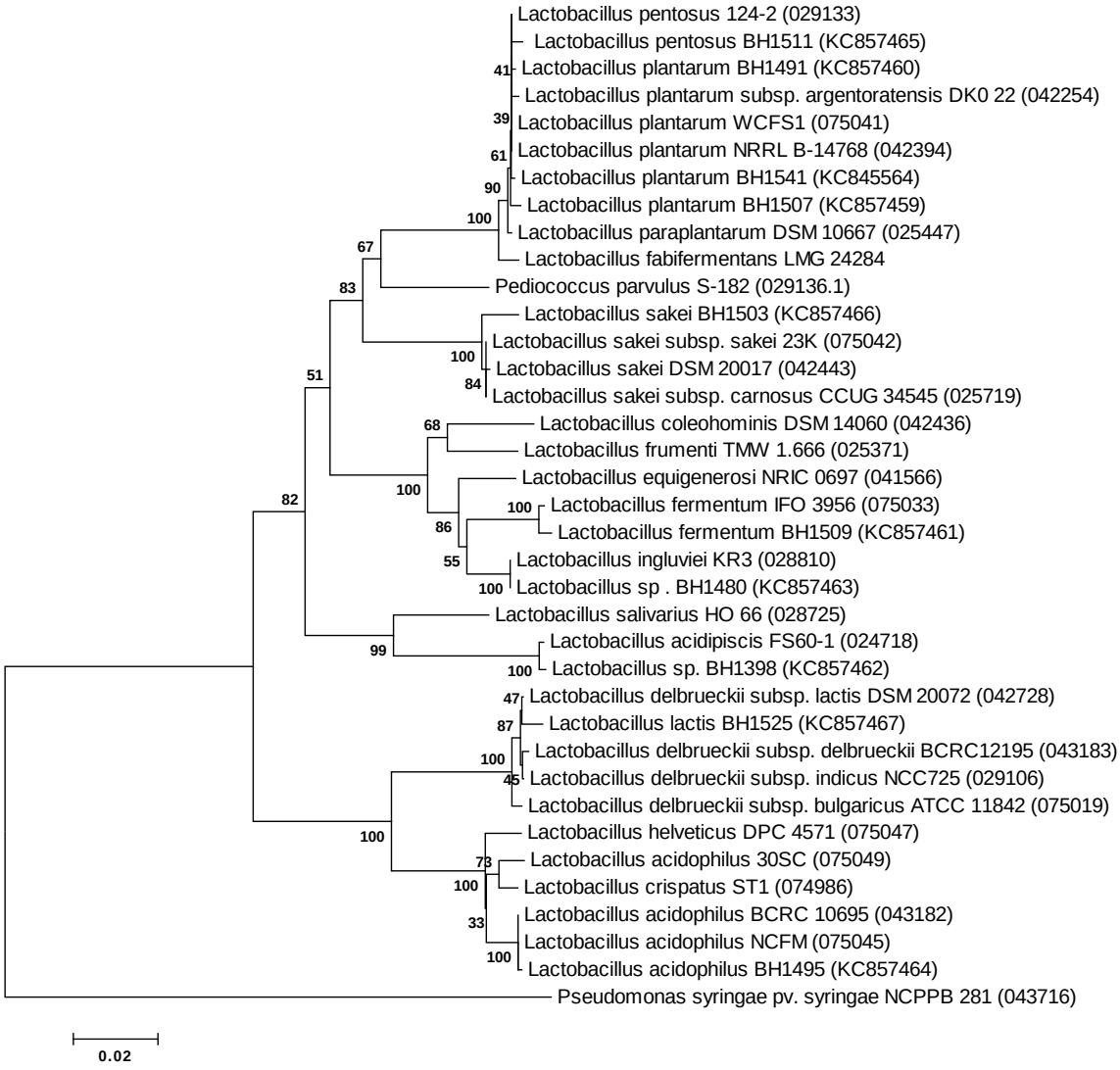


Figure 16: Phylogenetic tree based on 16S rDNA sequence analysis, showing the phylogenetic placement of selected LAB strains isolated from pollen grains. The tree was constructed by the neighbor-joining method, and *Pseudomonas syringae* pv. *syringae* was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

Furthermore, the 99 % similarity was shared between the isolate BH1541 and *Lactobacillus plantarum* WCFS1 (075041.1), *Lactobacillus plantarum* NRRL B-14768 (042394.1), *Lactobacillus plantarum* subsp. *argentoratensis* DK0 22 (042254.1), *Lactobacillus paraplantarum* DSM 10667 (025447.1), and *Lactobacillus pentosus* 124-2 (029133.1)

The strain BH1495 was more closely related (99% similarity) to *Lactobacillus acidophilus* NCFM (075045.1) and *Lactobacillus acidophilus* BCRC10695 (043182.1), than to *Lactobacillus crispatus* ST1 (074986.1) (97%), *Lactobacillus helv*eticus DPC 4571 (075047.1) (97%) and *Lactobacillus amylovorus* GRL 1112 (075048.1) (97%).

In this work, the strain BH1511 shares 98 % similarity with *Lactobacillus pentosus* strain 124-2 (029133.1) and *Lactobacillus plantarum* NRRL B-14768 (042394.1), as well as with *Lactobacillus paraplantarum* DSM 10667 (025447.1). For the strain BH1503, the strains *Lactobacillus sakei* subsp. *sakei* 23K (075042.1), *Lactobacillus sakei* DSM 20017 (042443.1), and *Lactobacillus sakei* subsp. *carnosus* CCUG 34545 (025719.1) were 97 % interrelated. By the same word, the strain BH1525 was genetically related (97%) to *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072 (042728.1), *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (075019.1), *Lactobacillus delbrueckii* subsp. *delbrueckii* BCRC12195 (043183.1), and *Lactobacillus delbrueckii* subsp. *indicus* NCC725 (029106.1). Tow other strains, BH1398 and BH1525, were related to *Lactobacillus acidipiscis* strain FS60-1 (024718.1) (97%) and *Lactobacillus ingluviei* strain KR3 (028810.1) (99%) respectively.

7.3.4 *Tolerance to low pH, bile acids, and hydrophobicity*

Then *lactobacillus* strains isolated from raw pollen samples were studied for their tolerance to unfavorable conditions (tolerance to acidic conditions and bile salts), which are essential traits as probiotics. Acid and bile tolerance were studied to predict the survival of probiotics after oral administration in vivo. The pH of gastric juice is often kept at 3 and pH 2 for 3 h is often used as an extreme condition to simulate the conditions in the stomach (Xiao-Hua *et al*., 2010). The results showed that low pH had obvious inhibition or killing effect on the viability of isolates (Tables 23). The viable counts of all isolates were significantly affected by the low acidity, especially at pH 1, 5. At this pH, the strains BH1398, BH1525, BH1480, and BH1503, could not survive for three hours. The other six strains, the mean Log CFU/ml decreases significantly from 9.71 to 1.73. However, strains of *Lactobacillus plantarum* and *Lactobacillus fermentum* were the most resistant, when compared to the other studied bacteria. At pH 2, only tow strains (*Lactobacillus* sp. BH1398 and *Lactobacillus* sp. BH1480) that could not recovered after exposure for three hours to this acidic condition. Among studied bacteria, *Lb. plantarum*, *Lb. fermentum*, *Lb. acidophyllus*, and *Lb. pentosus* proved to be the most resistant species, with losses in cell viability ranging from 0.77 to 4.04 log orders. In addition, at pH 3 all strains

could grow and resist the acidic conditions for three hours. The log CFU/mL ranged from 2.73 ± 0.33 to 9.30 ± 0.31, with losses in cell viability ranging from 0.40 to 3.61 log orders for most strains.

Tolerance to bile acids was assessed by measuring survival percentage after exposure the lactobacillus strains to three different concentrations of bile acids for 10 hours. It seems that, 0.3 % of bile acids does not affect greatly the survival of most strains, excluding *Lactobacillus* sp. BH1398. Survival ranged from 81.0 ± 3.5 to 93.5 ± 3.9%. Similarly, acceptable resistance was observed when these bacteria were cultured in the presence of 0.5% of bile salts, excluding *Lactobacillus* sp. BH1398. In contrast, in the presence of 1.0% bile salts, survival of five strains (*Lb. acidophilus* BH1495, *Lb. pentosus* BH1511, *Lactobacillus* sp. BH1398, *Lb. lactis* BH1525, and *Lactobacillus* sp. BH1480) was decreased by more than 50%. The other strains display moderate to good tolerance rate. In general terms, *Lactobacillus fermentum* BH1509 was considered the most tolerant strain (77.5% for 1% bile) followed by *Lactobacillus plantarum* BH1541 (59.9% for 1 % bile). In the present study, the highest hydrophobicity for hexadecane was observed in the isolates BH1507 (87.8%), BH1509 (78.1%), BH1541 (70.0%), and BH1491 (62.3%). While hydrophobicity of the other strains ranged from 16.1 to 48.9% (Table 24).

7.3.5 *Antibiotic susceptibility*

Results of the susceptibility of selected lactobacilli to ten antibiotics and tow proton pump inhibitors were shown in table 25. In general, resistance pattern was mostly distributed for all strains as compared to the data reported elsewhere (Charteris et al., 1998) and by EFSA (2005, 2008, 2010). However, four strains (BH1541, BH1495, BH1525, and BH1491) were sensitive to amoxicillin. Similarly, six strains were susceptibile to oxacillin. In contrast, penicillin was active against all strains. The strains, BH1480 and BH1491, were sensitive to Oxytetracyclin and Roxithromycin. This later antibiotic was also active against BH1541. In opposite, all strains were resistant to colistine, omeprazol, clindamycine, chloramphenicol, and ciprofloxacine. Furthermore, the tow protons pump inhibitors, metro-imidazole and ranitidine; have no effect on all strains (Table 25).

Table 23: Tolerance of ten *Lactobacillus* strains isolated from pollen grains to simulated gastric juice (Log CFU/mL).

Isolates	Resistance to gastric juice (log CFU/mL)			
	Initial mean counts	pH 1.5	pH 2	pH 3
Lactobacillus plantarum BH1541	9.70 ± 0.13	2.61 ± 0.17	8.76 ± 0.22	9.30 ± 0.31
Lactobacillus acidophilus BH1495	9.69 ± 0.12	1.42 ± 0.14	6.15 ± 0.26	8.66 ± 0.27
Lactobacillus plantarum BH1507	9.71 ± 0.23	2.13 ± 0.20	8.23 ± 0.20	8.93 ± 0.16
Lactobacillus fermentum BH1509	9.58 ± 0.11	2.84 ± 0.23	8.81 ± 0.18	8.33 ± 0.20
Lactobacillus pentosus BH1511	9.74 ± 0.20	1.87 ± 0.34	7.23 ± 0.32	8.10 ± 0.22
Lactobacillus sp. BH1398	9.70 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.73 ± 0.33
Lactobacillus lactis BH1525	9.82 ± 0.22	0.00 ± 0.00	4.50 ± 0.20	6.21 ± 0.15
Lactobacillus sp. BH1480	9.83 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	3.10 ± 0.15
Lactobacillus plantarum BH1491	9.56 ± 0.09	2.34 ± 0.15	6.53 ± 0.24	7.93 ± 0.18
Lactobacillus sakei BH1503	9.77 ± 0.18	0.00 ± 0.00	5.73 ± 0.30	7.40 ± 0.20

Table 24: Hydrophobicity and resistance of ten *Lactobacillus* strains isolated from pollen grains to bile salt.

Isolates	Bile Salt concentration			
	0.3%	0.5%	1%	Hydrophobicity (%)
Lactobacillus plantarum BH1541	87.4 ± 3.1	73.4 ± 2.1	59.9 ± 2.9	70.0 ± 1,33
Lactobacillus acidophilus BH1495	82.0 ± 2.9	72.5 ± 5.0	48.8 ± 3.0	46.8 ± 0,24
Lactobacillus plantarum BH1507	90.3 ± 3.5	88.7 ± 3.4	76.4 ± 2.8	87.8 ± 1,52
Lactobacillus fermentum BH1509	91.5 ± 4.6	83.4 ± 4.0	77.5 ± 3.9	78.1 ± 0,45
Lactobacillus pentosus BH1511	93.5 ± 3.9	69.5 ± 3.9	39.1 ± 2.8	21.1 ± 0,21
Lactobacillus sp. BH1398	49.2 ± 3.1	39.0 ± 3.0	21.7 ± 2.9	48.9 ± 0,28
Lactobacillus lactis BH1525	75.0 ± 5.1	57.7 ± 5.2	37.0 ± 3.6	46.7 ± 0,14
Lactobacillus sp. BH1480	83.3 ± 2.7	68.3 ± 3.0	31.6 ± 3.8	16.1 ± 1,23
Lactobacillus plantarum BH1491	89.6 ± 4.8	74.9 ± 4.5	53.3 ± 4.1	62.3 ± 0,35
Lactobacillus sakei BH1503	81.0 ± 3.5	61.4 ± 3.7	57.6 ± 3.4	29.9 ± 0,58

Table 25: Antimicrobial susceptibility of ten *Lactobacillus* strains to drugs used for the treatment of gastritis and gastro-duodenal inflammation.

Strains	BH1541	BH1495	BH1507	BH1509	BH1511	BH1398	BH1525	BH1480	BH1491	BH1503	*MIC Break-point
Amoxicillin	S	S	R	R	R	R	S	R	S	R	16
Colistine	R	R	R	R	R	R	R	R	R	R	4
Omeprazol	R	R	R	R	R	R	R	R	R	R	/
Oxacilline	R	R	S	S	S	R	S	R	S	S	32
Clindamycine	R	R	R	R	R	R	R	R	R	R	1-2
Chloramphenicol	R	R	R	R	R	R	R	R	R	R	64
Ciprofloxacine	R	R	R	R	R	R	R	R	R	R	32
Roxithromycin	S	R	R	R	R	R	R	S	S	R	16
Oxytetracyclin	R	R	R	R	R	S	S	S	S	R	4-32
Penicillin G	S	S	S	S	S	S	S	S	S	S	32
Metro-imidazole	R	R	R	R	R	R	R	R	R	R	/
Ranitidine	R	R	R	R	R	R	R	R	R	R	/

* Break-point values (µg/ml) were from EFAST (2005), EFAST (2008), EFAST (2012), and Charteris *et al*. (1998)

S, Susceptible; R, Resistant; /, Not available;

7.4 DISCUSSION

7.4.1 *Antimicrobial activity*

The results of antimicrobial activity partially confirm those of Nieto-Lozano *et al*. (2002) who found that strains of Lactobacilli had inhibitory activity against *Staphylococcus aureus* and not against gram negative bacteria. Results of this work were in line with data reported by Essid *et al*. (2009). However, Klinberg *et al*. (2005) reported that *Lactobacillus plantarum* strains, isolated from fermented sausages, had inhibitory activity against *E. coli* and *Salmonella typhimirium*. It was reported that strains of *Lactobacillus plantarum* could inhibit Gram-positive bacteria (*Staphylococcus aureus*) better than Gram-negative bacteria *Salmonella arizonae*, *E. coli*, and *Pseudomonas aeuroginosa* (Albano *et al*., 2007; Ammor *et al.*, 2005). It seems that (for some researchers) the outer membrane of Gram-negative bacteria may protect the cytoplasmic membrane from the action of antimicrobial compounds (Gao *et al*., 1999). Other than organic acids and hydrogen peroxide, lactobacilli produce bacteriocin-like molecules which were active against Gram-positive and Gram-negatibe bacteria. The biosynthesis of other low molecular compounds (aromatic nature) was reported for lactobacilli (Niku-Paavola *et al*., 1999).

7.4.2 *Phenotypic and Genotypic Characterization*

Fermentative profiles for studied pollen associated lactobacilli are usually determined by API 50 CH System and other physiological traits. Based on this characterization, the fermentative capacity of *lactobacillus* species profiles may vary from a very few carbon sources (e.g., *Lb. sanfranciscensis*) (Foschino *et al*., 2001), to a very broad range of substrates (e.g., *Lb. plantarum*) **(**Hammes and Vogel, 1995). Overall, a striking property of pollen *lactobacillus* is their enormous flexibility and potential to catabolic substrates as a response to biochemical complexe composition in the surrounding environment. Pollen carbohydrates may reaches up to 55% of dray weight. However, fructose, glucose and sucrose comprise about 90% of all sugars. Other high molecular carbohydrates like starch and cellulose are main pollen cell components (Serra-Bonvehi *et al.*, 1986). The majority of strains have the capacity to ferment N-acetyl-d-glucosammine, fructose, glucose and maltose. Maltose, glucose and fructose are the carbon sources present at sufficient concentrations in pollen grains. Pollen carbohydrate content varies widely in pollen. Todd and Bretherick (1942) recorded values from 1% to 37% of total dry mass in hand-collected pollen, and from 21% to 48% in bee-collected pollen. Particularly because of the added nectar, carbohydrate constitutes a large fraction of the nutritional content of pollen. In other reports, carbohydrates reach more than 55 % of total pollen dry masse (Serra-Bonvehi *et al.*, 1986). It seems that pollen associated bacteria were physiologically adapted to sugars present in pollen grains. Regarding other potential substrates available in pollen, all strains used variousely mannitol and pentoses such as xylose, ribose and arabinose, as these sugars are liberated in after enzymatic

hydrolysis of pentosans and other complex carbohydrates present in pollen grains. Fermentation of pentoses by heterofermentative strains takes place through the lower half of the 6-phosphogluconate pathway, and, in this case, only one mole of NADH + H+ has to be oxidized. There is, therefore, no need to form ethanol; instead, an extra mole of ATP and acetate is formed from acetyl-phosphate (Gobbetti *et al*., 2005). Only some strains belonging to species *Lb. hammesii* (Valcheva *et al*., 2005), *Lb. spicheri* (Meroth *et al*., 2004), *Lb. brevis* and *Lb. plantarum* (Gobbetti *et al*., 2000) seemed to have the same capacity. Arabinose, glucose, fructose, mannose, mannitol, gentiobiose, melezitose, melibiose, saccharose, trehalose, were variously fermented. These carbon sources correspond mainly to those prevalent in plant kingdoom (Buckenhüskes, 1997). Similar phenotype profiles were found for strains of *Lb. plantarum* isolated from Thai fermented fruits and vegetables (Tanganurat *et al*., 2009; Cagno *et al*., 2010). Glycoside such as amygdalin was not used only by isolates of BH1495, BH1509, and BH1503. arbutin was used by isolates of all clusters, and salicin was not used only by tow isolates (BH1495 and BH1509). Even these glycosides are typically found in vegetables. Starch was hydrolysed only by the strain. This polymer is a reserve polysaccharide in pollen grains. According to the fermentation profile, it seems that the isolates assimilate variously a panel of carbohydrates which reflects their enzymatic and genetic potentials. Furthermore, these traits were shared with lactobacillus species from dairy or animal origin and other natural plant related ecosystems.

The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis of phenotypical features do not correspond with their phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR)-based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE) are regarded important for the specific characterization and detection of LAB strains (Mohania *et al*., 2008). Specifically, many studies emphasize that the classification of lactobacilli is unsatisfactory and does not reflect the real phylogenetic relatedness of different strains and species. Several new genetic and chemotaxonomic approaches have been used during the last 29 years with an aim of improving the classification and identification of lactobacilli. However, analysis of plasmid content, sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of whole-cell protein and of total soluble cell protein, sequencing of rRNA, restriction endonuclease fingerprinting, and DNA-DNA hybridization. All of these approaches have improved the taxonomic knowledge of the generic and suprageneric relationships of lactobacilli (Zhong *et al*., 1998). It is well known that the species *Lactobacillus plantarum, Lactobacillus pentosus*, and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes. In the present results, miss identification of the isolates BH1507, BH1491, BH1541 and BH1511 to a defined species was encountered. These strains share 99% and 98% (for the later strain) similarity with three different *Lactobacillus*

species (*Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum*). The genetic heterogeneity of the *Lb. plantarum* group has been demonstrated on the basis of DNA-DNA hybridization data and three groups were identified which were later classified as *Lb. plantarum* sensu stricto, *Lb. pentosus*, and *Lb. paraplantarum*. Moreover, Torriani *et al*. (2001) separate the three species by recA Gene Sequence Analysis and Multiplex PCR Assay with recA Gene-Derived Primers. It is believed that universal 16S rRNA gene sequencing alone was not efficient to separate the three species. In other studies, combination of 16S rRNA-ARDRA leads to a beter separation of *lactobacillus* species (Rodas *et al*., 2003). By the same word, 16S rRNA gene sequencing does not differentiate between subspecies of *Lactobacillus sakei* and *Lb. delbrueckii*. In fact, pecific PCR and randomly amplified polymorphic DNA PCR (RAPD-PCR), were used for rapid and reliable differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*. Using this strategy by Torriani *et al*. (1999), two PCR-based systems were available for rapid identification and differentiation of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*; specific amplification with primers LB1 and LLB1, which target the pepIP gene sequence of *Lb. delbrueckii* subsp. *bulgaricus*, and a RAPD-PCR assay with primer M13, which can be used either separately or in combination for greater reliability.

7.4.3 *Tolerance to unfavorable conditions*

Different regions of the gastrointestinal tract have varying acid levels. Stomach and the regions after stomach have the highest acidity and the pH of these areas may fall to as low as pH (1.5). Probiotic microorganisms need to resist the adverse factors in the gastrointestinal tract when they pass through it, like the stomach acidity and bile salts, excreted in duodenum (Jin *et al*., 1998). To assure that bacteria arrive in suitable concentration (6 to 8 logarithms/g of consumed food) to the stomach and intestine, and exert their probiotic effect (Shah *et al*., 1999), In order to be used as beneficial adjuncts, *Lactobacillus* must be able to survive these harsh conditions and colonise in the gut. Therefore, it was decided to select strains with highest resistance in this investigation.

7.4.3.1 *Acid tolerance*

Over 2 litres of gastric juice, with a pH as low as 1.5, is secreted by parietal cells into the stomach each day (Morelli, 2000), which causes the destruction of most microorganisms ingested (Kimoto e*t al*., 2000). Hydrochloric acid, the main constituent of gastric juice, helps destroy ingested microorganisms and stimulates the activation of pepsin, which degrades proteins into peptides. In conjunction, these secretions provide a powerful barrier to the survival of ingested probiotic bacteria. In this sense, resistance to human gastric transit is an important selection criterion for probiotic microorganisms (Charteris *et al*., 1998). Species of the genus *Lactobacillus* have long been regarded as acid tolerant; however, gastric resistance has been shown

to be highly strain-dependent, with great variations between strains of the same species (Tannock, 2004). The results of these tests are predictive of the ability of the strains to survive in gastric acidic conditions. Garriga *et al*. (1998) screened lactic acid bacteria with regard to pH 3 tolerance, and showed that pH 3 did not decrease the number of lactic acid bacteria. It has been reported (Garriga *et al*., 1998; Charalampopoulos and Pandiella, 2010), that *Lb. plantarum* NCIMB 8826 strain was shown to be acid tolerant and has been shown to exert anti-inflammatory activities in animal models (Foligne *et al*., 2006).

Acid survivability of the strain *Lb. acidophilus* (Liong and Shah, 2005), and lactic acid producing bacteria (Thirabunyanon *et al*., 2009; Hossain *et al*., 2012) have been previously reported. Remarkable acid tolerance and survivability rates were obtained in this experiment using *lactobacillus* strains from pollen (a bee hive product). This tolerance may be due to adaptation of the strains to the known acidity of pollen grains. In the present results, lower pH values (1.5 and 2.0) affect greatly bacterial cell viability. One explanation that this might be due to the fact that at a pH lower than the pKa value of organic acids (derived from food and from gut), a major portion of the acid is in the non-ionized form, which is permeable through the cell membrane of microbes. Once non-ionized acid is inside the cell where the pH is near neutral, acid ionizes and hence, cannot come out of the cells (Levine and Fellers, 1940; Warth, 1977). Therefore, at lower pH there is a constant influx of organic acids into the cells which ultimately results in death. These results are in agreement with (Coppola *et al*., 2005), who selected *Lb. rhamnosus* strain isolated from Parmigiano Reggiano cheese based on their survivals after 2 and 4 hours of incubation at pH 3.0, which was similar to the study performed by Chou and Weimer (1999) on *Lb. acidophilus*. Prasad *et al*. (1998) obtained four acid tolerant strains from 200 LAB isolates based on their 80% survival after exposure to pH 3 for 3 hours. In addition, Buntin *et al*. (2008) were selected acid tolerant 32 strains isolated from gastrointestinal tracts of marine fish. This higher resistance of *Lactobacillus planta*rum, *Lb. acidophilus*, *Lb. fermentum*, *Lb. sakei* and *Lb. pentosus* to simulated gastric conditions may be attributable to elevated enzyme F0F1-ATPase activity which has been shown to be present in certain *Lactobacillus* strains (Corcoran *et al*., 2005). In general, the acid tolerance of lactobacilli is attributed to the presence of a constant gradient between extracellular and cytoplasmic pH. When the internal pH reaches a threshold value, cellular functions are inhibited and the cells die. The F_0F_1-ATPase is a known mechanism that gram-positive organisms use for protection against acidic conditions (Kashket, 1987; Cotter and Hill, 2003). The F_0F_1-ATPase is a multiple-subunit enzyme consisting of a catalytic portion (F1) incorporating the α, β, γ, δ, and ξ subunits for ATP hydrolysis and an integral membrane portion (F0) including the a, b, and c subunits, which function as a membranous channel for proton translocation (Sebald *et al*., 1982). The role of the F_0F_1-ATPase in organisms devoid of a respiratory chain is to generate a proton motive force, via proton expulsion. As a consequence, it is thought that the F_0F_1-ATPase can increase the

intracellular pH at a low extracellular pH. F_0F_1-ATPase is induced at low pH, and regulation appears to occur at the transcriptional level (Fortier *et al*., 2003).

7.4.3.2 *Bile tolerance*

Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile and the concentration of bile in different regions of the intestine vary, depending mainly on the type of food consumed and it may not be possible to predict the bile concentration in the intestine at any given moment. Davenport (1997) reported that while bile concentrations in the intestine range between 0.5 to 2.0% during first hour of digestion. The levels may decrease during the second hour. Bile concentrations ranging from 0% to 1.5% have been used in several microbiological media for selective isolation of bile tolerant bacteria from mixed cultures. Gilliland *et al*. (1984), Goldin et al., (1992) considered 0.3% bile salts as a critical concentration for the screening of resistant strains. The ten lactobacillus strains were selected to test their ability to survive in simulated small intestinal juice with three different bile concentrations (0.3, 0.5, and 1.0%). It was considered that 0.45% of bile is sufficient to determine any resistant strains (Buntin *et al*., 2008). As shown in Table 24, 50% of strains survived simulated small intestinal juice with 1.0% bile salt for ten hours with percent cell viablity more than 50%. Tow strains (BH1507 and BH1509) were observed to be the most bile tolerant with survival rates of 76.4 and 77.5 %, respectively. The viability of *Lb. plantarum* NCIMB 8826 isolated from human saliva was similarly found to decrease by 1.9 Log CFU/mL (Patel *et al*., 2004). Our findings are in accordance to the result obtained by Erkkila and Petaja (2000) with the strains of *Pediococcus acidilactici* P2, *Lactobacillus curvatus* RM 10 and *Lactobacillus sake* L2 were the most resistant to 3 % bile salt at pH 6. Pennacchia *et al*. (2004) reported that the bile salt tolerance of the *Lactobacillus* strains were able to grow in media supplemented with 3% bile salt. However, *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese were able to survive at bile salt concentration of 1, 1.5 and 2 % after 48 hour of incubation at 37°C (Coppola *et al*., 2005). According to Serrazanetti *et al*. (2009), the small intestinal juice tolerance of probiotic bacteria was strain dependent. Bile resistance of *Lactobacillus* spp. is related to the specific enzyme activity of bile salt hydrolase (BSH) which helps the hydrolysis of conjugated bile and thus reduces its toxic effects (Du Toit *et al*., 1998; Erkkila and Petaja, 2000), which are then readily excreted from the GI-tract (Maragkoudakis *et al*., 2006). This particular enzyme decreases bile solubility and thus weakening its detergent effect.

7.4.4 *Antibiotic susceptibility*

Safety confers another essential property of probiotic bacteria. These GRAS alleviated microorganisms are powerful dietary supplements that help human and animals rebuild the balance of beneficial micro organisms in their gastrointestinal tract and reverse the imbalances that may contribute to the onset of chronic

conditions. In this way, the food industry will need to carefully assess the safety and efficacy of all new species and probiotic strains before their incorporation into food products (Parvez *et al*., 2006). According to the EFSA's guidance, micro-organism showing MICs same or less than EFSA's breakpoint for a specific antimicrobial is defined as susceptible to this antimicrobial (EFSA, 2008). In the present research, resistance to oxacillin and amoxicillin was shared by nearly 50% of the isolates. The resistance of all isolates to clyndamycin and chloramphenicol and the resistance patterns of four strains to oxytetracyclin were comparable to the data reported elswere (Temmerman *et al*., 2003; Coppola et al., 2005; D'Aimmo *et al*., 2007; Jamaly *et al*., 2011). In general, glycopeptide, aminoglycoside and sulfamethoxazole resistance has been formerly described in LAB species (Elisha and Courvalin, 1995; Elkins and Mullis, 2004), and in all cases it has been associated with their natural and intrinsic resistance due to membrane impermeability, probably complemented by potential efflux mechanisms resistance (Elkins and Mullis, 2004). Intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria (Mathur and Singh, 2005).

Chapter 08

Antibiotic Susceptibility of Lactobacilli

8. Antibiotic Susceptibility of *Lactobacillus* Strains Isolated From Pollen Grains

8.1 INTRODUCTION

The major financial and societal costs caused by the emergence and evolution of antibiotic resistance in pathogenic bacteria represent a well-known problem. Reports suggest that commensal bacteria may act as potential reservoirs for antimicrobial resistance genes, hence bacteria used as probiotics for humans or animals should not carry any transferable antimicrobial resistance genes (Ashraf and Shah, 2011; Jacobsen *et al.*, 2007). Antibiotic resistance of LAB used for food, feed and probiotic applications has been proposed as a hazard due to the potential risk for transfer to pathogenic bacteria (Flórez *et al*., 2006). According to World Health Organization (WHO) global strategy for the containment of antimicrobial resistance (WHO, 2001), the rate of emergence of antimicrobial resistance is expected to be increased by misuse of antibacterial substances. The European Food Safety Authority (EFSA, 2005) has outlined a scheme based on the qualified presumption of safety (QPS) that involves the individual assessment and evaluation of acquired antibiotic resistance determinants in lactic acid bacteria (LAB). In the EFSA guidelines (EFSA, 2008), the MICs for relevant antimicrobials have been set for the following genera (and in some cases individual species): *Lactobacillus*, *Lactococcus*, *Streptococcus thermophilus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Propionibacterium*, *Bifidobacterium* and *Bacillus*. These genera also cover the recent QPS lists for bacteria, and consequently the FEEDAP approach can be directly applied (Ashraf and Shah, 2011; EFSA, 2012). The two major applications of lactobacilli are as starter cultures of food and feed and as probiotics. Specific strains of e.g. *L. casei, L. johnsonii, L. rhamnosus, L. plantarum* and *L. reuteri* have been shown to be protective against a variety of gastrointestinal infections and allergic disorders (Saxelin *et al*., 2005; Britton and Versalovic, 2008). However, the mechanisms of action of these bacteria are just beginning to be understood. Putative probiotic mechanisms are related to production of antimicrobial compounds, interference with pathogens in terms of competition for nutrients or mucosal attachment, enhancement of intestinal barrier function and immunomodulation (Saxelin *et al.*, 2005; Britton and Versalovic, 2008; Egervärn *et al*., 2009). Antibiotic resistance in lactobacilli has been heavily debated during the last decade. This situation is supported by the fact that the same type of genes encoding resistance to, for example, tetracycline, erythromycin, chloramphenicol, streptomycin and streptogramin have been found in commensal lactococci and lactobacilli as well as in potentially pathogenic enterococci and pathogenic streptococci (Teuber *et al.*, 1999). Lactobacilli are generally recognized as safe, and are industrially important food-grade organisms used as probiotics and starter cultures in fermented foods. They are present in ready-to-eat foods and are also indigenous members of the human intestinal microbiota. Lactobacilli that harbor antibiotic resistance determinants have been found in a broad selection of food products (Herrero *et al*., 1996; Gevers *et al.*, 2000; Temmerman *et al*., 2003; Florez *et al*., 2005). Owing to their wide environmental distribution, it is

possible that these commensal bacteria act as vectors for the dissemination of antibiotic resistance determinants via the food chain to the consumer, a risk that has so far been poorly addressed. This paper reports the susceptibility patterns of a candidate probiotic *Lactobacillus* species isolated from row pollen. This work was performed to select strains that do not contain antibiotic transferable resistances among those with desirable technological and probiotic characteristics.

8.2 MATERIALS AND METHODS

8.2.1 *Bacterial strains, media, and culture conditions*

Lactobacillus strains used were previously isolated from raw pollen grains and characterized based on phenotypic features (Belhadj *et al*., 2014). 13 strains of *Lactobacillus plantarum,* two *Lactobacillus lactis*, 16 *Lactobacillus fermentum*, 12 *Lactobacillus sp.*, 08 *Lactobacillus acidophilus*, 04 *Lactobacillus pentosus*, 13 *Lactobacillus sakei* were used in this investigation. The strains were grown in MRS agar (Fluka) at 35 °C anaerobically for 24–48 h. Bacterial suspensions were prepared by suspending one to three colonies from MRS plates in sterile saline solution (0.85 % NaCl). The solutions were equilibrated to 0, 5 McFarland equivalent turbidity.

8.2.2 *Determination of the minimum inhibitory concentration*

For *Lactobacillus* isolates, the MICs of 12 antibiotics were assessed using the standardized broth micro-dilution technique of the National Committee for Clinical Laboratory Standards (NCCLS) in MRS (Fluka) broth (NCCLS, 2007). The antibiotics analyzed (Table 26 included inhibitors of cell-wall synthesis (the β-lactams penicillin G, amoxycillin, gentamici; the cephalosporin cefoxitin; streptomycin, and the glycopeptides vancomycin), protein synthesis (chloramphenicol, clindamycin, and tetracycline), and nucleic-acid synthesis (the fluoroquinolone moxifloxacin; and metronidazole). Briefly, colonies from MRS agar were used to make an even suspension (equivalent to the 0.5 McFarland turbidity standard) in sterile saline solution (085 % NaCl). Fifty-microlitre aliquots of this suspension were inoculated into each well of the 96 bottomed wells microtiter plate containing fifty microliter of double strength MRS broth (pH, 6.8). Plates were incubated anaerobically at 35 °C for 24-48 h.

Table 26: Antimicrobials included in the micro-dilution test and their range of dilutions used to determine the minimum inhibitory concentrations.

Antibiotic	Type of antibiotic	Range of dilutions (µg/mL)
Inhibitors of cell-wall synthesis		
Penicillin G	Penicillin	0.125–256
Amoxicillin	Penicillin	0.125–256
Gentamycin		0.125-128
Imipenem	Carbapenems	0.06–128
Cefoxitin	Cephalosporin	0.25–512
Sytreptomycin		025-256
Vancomycin	Glycopeptide	2–512
Inhibitors of protein synthesis		
Chloramphenicol		025–256
Tetracycline		1–512
Clindamycin	Lincosamide	0.25–256
Inhibitors of DNA synthesis		
Moxifloxacin	Fluoroquinolone	0.122–128
Metronidazol		0.5–256

8.3 RESULTS

The complete distribution of MICs of 12 antimicrobial agents tested for 68 *Lactobacillus* isolates was examined (Tables 27 and 28). MICs for the *Lactobacillus* species for cell-wall-synthesis inhibitors are summarized in Table 27. The highest MICs for penicillin were shown to be ≤4 μg/mL. The highest for amoxycillin and imipenem were ≤16 μg/mL and 4μg/mL repectively. The MICs for all cell-wall-synthesis inhibiting antibiotics were not very high. The penicillins tested in this study exhibited comparable antimicrobial activities against all *lactobacillus* species examined. In general, most of the isolates were inhibited by a maximum of 4 or 16 μg/mL of the corresponding penicillin. However, isolates of *Lactobacillus lactis* and *Lactobacillus pentosus* were relatively more susceptible to penicillins in comparison with other tested species. The *L. acidophilus* strains were also relatively more susceptible to penicillins than to aminopenicillins when compared with *L. plantarum*, *L. fermentum* and, to a lesser extent, also with *L. sakei*.

For cefoxitin, great variation of susceptibility was noted. MICs range between 1 μg/mL and 256 μg/mL, and 45.58 % of the isolates were susceptible to this antibiotic (MIC ≤16 μg/mL). However, strains of *Lactobacillus lactis* and *Lactobacillus pentosus* were more sensitive. In contrast, strains of *Lactobacillus fermentum*, *Lactobacillus* sp. and in some extent *Lactobacillus plantarum* were the resistant ones (Table 27).

The aminoglycosides gentamicin and streptomycin showed well-defined MIC distributions between ≤0.25 and 16 μg/mL for gentamicin and between ≤1 and 128 μg/mL for streptomycin. However, most isolates displayed MICs at the low end of the concentration ranges (≤250 μg/mL for gentamicin and ≤1 μg/mL for streptomycin, respectively). Strains of *L. acidophilus* were relatively more susceptible to streptomycin than other *Lactobacillus* species. In contrast, members of *Lactobacillus plantarum* and, to some extent also *L. fermentum* and *L. sakei*, appeared to be less susceptible to streptomycin. It was found that 76.47 % of *Lactobacillus* strains (52 strains) were resistant to high concentrations of vancomycin (the majority of MICs were ≥ 256 μg/mL).

With the exception of vancomycin, the MICs of antibiotics affecting the synthesis of proteins showed the greatest variation between species and strains (Table 28). For all other antibiotics of this group (chloramphenicol, clindamycin, and tetracycline), most strains were clearly moderate to strongly resistant strains were seen (Table 28). Some strains proved resistant to more than 1 of these antibiotics. For instance, 20 isolates were resistant to chloramphenicol (MIC ≥32 μg/mL), 8 isolates were resistant to clindamycin (MIC ≥ 128 μg/mL), and more than 50 % of isolates were resistant to tetracycline (MIC ≥ 64 μg/mL). However, *Lactobacillus lactis* isolates were found to be sensitive to the three antibiotics affecting protein synthesis (MIC ≤ 32 μg/mL) (table 28).

Table 27: Distribution of MICs to several antibiotics inhibiting cell-wall synthesis (the β-lactams penicillins, cephalosporins, and carbapenems; and the glycopeptide vancomycin) for *Lactobacillus* species isolated from pollen grains.

	Species (No. of strains)	Number of isolates for which the MIC (µg/mL) was as follows												
		≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥256
Penicillin G	*Lactobacillus plantarum* (13)		3	4	2	1	3							
	Lactobacillus lactis (02)		2											
	Lactobacillus fermentum (16)	2	4	3	7									
	Lactobacillus sp. (12)		2	2	3	1	4							
	Lactobacillus acidophilus (08)	1	2	1		4								
	Lactobacillus pentosus (04)	1	2	1										
	Lactobacillus sakei (13)		2	3	5	1	2							
Amoxicillin	*Lactobacillus plantarum* (13)		1		3	3	2	4						
	Lactobacillus lactis (02)			2										
	Lactobacillus fermentum (16)		1	6	2	1	4	1	1					
	Lactobacillus sp. (12)		2	4	1	1	3		1					
	Lactobacillus acidophilus (08)		1		1	2	3	1						
	Lactobacillus pentosus (04)			3		1								
	Lactobacillus sakei (13)	3	4	2	1		3							
Gentamicin	*Lactobacillus plantarum* (13)			1	3	2	3	4						
	Lactobacillus lactis (02)			2										
	Lactobacillus fermentum (16)		2	1	4	5	1	2	1					
	Lactobacillus sp. (12)		1	4	1	3	2		1					
	Lactobacillus acidophilus (08)			1	2	2	1		2					
	Lactobacillus pentosus (04)		1	2	1									
	Lactobacillus sakei (13)			2	1	1	3	4	2					
Imipenem	*Lactobacillus plantarum* (13)	3	2	4	3	1								
	Lactobacillus lactis (02)				2									
	Lactobacillus fermentum (16)	1	2	4	5	1								
	Lactobacillus sp. (12)	3	1	2	3	2								
	Lactobacillus acidophilus (08)	1	2	4	1									
	Lactobacillus pentosus (04)	3	1											
	Lactobacillus sakei (13)		2	5	1	2	3							
Streptomycin	*Lactobacillus plantarum* (13)						2	3	1	4	2	1		
	Lactobacillus lactis (02)					2								
	Lactobacillus fermentum (16)					3	1	5	2	1	3			
	Lactobacillus sp. (12)				1	3	4	2	2					
	Lactobacillus acidophilus (08)					3	1	4						
	Lactobacillus pentosus (04)					2	2							
	Lactobacillus sakei (13)					1	2	5	4	1				
Cefoxitin	*Lactobacillus plantarum* (13)					1	2	1	4	2	1	2		
	Lactobacillus lactis (02)						1		1					
	Lactobacillus fermentum (16)				1	1		2	3	1	1	3	4	
	Lactobacillus sp. (12)							1	2	2	1	3	3	
	Lactobacillus acidophilus (08)							2	1	1	2	2		
	Lactobacillus pentosus (04)							1		3				
	Lactobacillus sakei (13)						3	2	1	2	1	3	1	
Vancomycin	*Lactobacillus plantarum* (13)													13
	Lactobacillus lactis (02)											1	1	
	Lactobacillus fermentum (16)												2	14
	Lactobacillus sp. (12)													12
	Lactobacillus acidophilus (08)										2	4	2	
	Lactobacillus pentosus (04)												4	
	Lactobacillus sakei (13)													13

Table 28: Distribution of MICs to several antimicrobials inhibiting protein synthesis and those inhibiting the synthesis of DNA for *Lactobacillus* species from pollen grains.

	Species (No. of strains)	Number of isolates for which the MIC (µg/ml) was as follows												
		≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥256
Chloramphenicol	*Lactobacillus plantarum* (13)				2	1	4	3	1	2				
	Lactobacillus lactis (02)						1		1					
	Lactobacillus fermentum (16)				3	2	4	1	2	1	3			
	Lactobacillus sp. (12)						2	3	4	1	2			
	Lactobacillus acidophilus (08)					2	1			5				
	Lactobacillus pentosus (04)					1			1	2				
	Lactobacillus sakei (13)				1	2	1	1	4	4				
Clindamycin	*Lactobacillus plantarum* (13)					2	3	1	3		2	1		1
	Lactobacillus lactis (02)				2									
	Lactobacillus fermentum (16)				2	1	2	4	2	3		2		
	Lactobacillus sp. (12)					2	1		3	4		2		
	Lactobacillus acidophilus (08)			3	1			2	2					
	Lactobacillus pentosus (04)							1	3					
	Lactobacillus sakei (13)				1	3	2		1	4		2		
Tetracycline	*Lactobacillus plantarum* (13)							2	1	2	3		1	4
	Lactobacillus lactis (02)								1	1				
	Lactobacillus fermentum (16)							1		3	2	1	4	5
	Lactobacillus sp. (12)						1	2	1	1	1	2	4	
	Lactobacillus acidophilus (08)						2	1	3	2				
	Lactobacillus pentosus (04)						2	1	1					
	Lactobacillus sakei (13)								3	1	3	1	5	
Moxifloxacin	*Lactobacillus plantarum* (13)			3	1	1	5	4	1					
	Lactobacillus lactis (02)				2									
	Lactobacillus fermentum (16)		1	2	4	1	4	1		3				
	Lactobacillus sp. (12)			3	4	1	2	1	1					
	Lactobacillus acidophilus (08)				4	1	2	1						
	Lactobacillus pentosus (04)			1		3								
	Lactobacillus sakei (13)				3	1	5	2	1	1				
Metronidazole	*Lactobacillus plantarum* (13)								1	2	4	1	2	3
	Lactobacillus lactis (02)							2						
	Lactobacillus fermentum (16)						3	1	1	2	4	1	4	2
	Lactobacillus sp. (12)							4	1	1	3	1	2	
	Lactobacillus acidophilus (08)					1		1	4	1	1			
	Lactobacillus pentosus (04)								1	3				
	Lactobacillus sakei (13)							3	1	4	1	2	1	

Four *Lactobacillus plantarum* isolates and five from *Lactobacillus fermentum* were resistant to tetracyclin (MIC ≥ 256 μg/mL), 6 of which were resistant to chloramphenicol (MIC ≥ 32 μg/mL). Also, *Lactobacillus acidophilus* seems to be more susceptible to these antibiotics more than the other species. From all isolates, 80 % were resistant to Metronidazole (MIC ≥ 32 μg/mL). Three isolates of *Lactobacillus plantarum* and two *Lactobacillus fermentum* were strongly resistant to this antibiotic (MIC ≥ 256 μg/mL) (Table 28). Only few variations were observed among studied isolates. In contrast, isolates display susceptibility pattern to moxifloxacin (MIC ≤ 32 μg/mL), and the majority of MICs (89.70 %) were ≤ 8 μg/mL. Furthermore, three isolates of *Lactobacillus fermentum* and one *Lactobacillus sakei* were relatively moderate resistant to this antibiotic (MIC = 32 μg/mL).

8.4 DISCUSSION

In the present study, MICs of 12 antimicrobial agents of nearly all important classes were determined for a collection of 68 isolates encompassing 6 species of the genus *Lactobacillus*, using MRS medium and micro-broth dilution assay according to NCCLS recommendations. The MICs for different antibiotics seem to be strain-specific. However, differences may also be a result of the different methods being used, such as the E-test, agar dilution, disk diffusion, and microbroth methods (Florez *et al*., 2005), although various studies have been reported that different nutrient media, incubation conditions and/or susceptibility testing methods may affect end results and variability of MICs were recorded (De la Maza *et al*., 1989; Danielsen and Wind, 2003; Gevers *et al*., 2003; Perez-Pulido *et al*., 2005). Furthermore, a variety of different methods has been used and no standard methods are proposed. In some studies, (Charteris *et al*., 2001; Danielsen and Wind, 2003) higher MIC values for streptomycin and gentamicin were recorded. It seems that the increased MICs obtained was ascribed for medium pH (MRS, pH 6.2 ± 0.2) because the pH optimum of aminoglycosides is in the alkaline range (pH 7.8) (Amsterdam, 2005), which was not in accordance with our results, where, the MRS's pH was adjusted to 6.8, which appears to favor the antibacterial activities of aminoglycosides, resulting in lower MICs. However, resistance against aminoglycosides, such as neomycin, kanamycin, streptomycin and gentamicin has been observed more frequently among lactobacilli (Danielsen and Wind, 2003; Coppola *et al*., 2005 Zhou *et al*., 2005). The medium of choice has been demonstrated to significantly affect to the classification of LAB as being either susceptible or resistant (Zhou *et al*., 2005), especially with aminoglycosides and MRS medium. Lactobacilli are generally susceptible to penicillins, and more resistant to cephalosporins (Huys *et al*., 2002; Danielsen and Wind, 2003). The resistance mechanism is not fully elaborated, but cell wall impermeability and non-specific multidrug transporters may be involved (Ammor *et al*., 2007). In general, lactobacilli are generally susceptible to all protein

synthesis inhibitors except aminoglycosides. Intrinsic resistance to the latter group of antibiotics is attributed to the absence of cytochrome-mediated electron transport, enabling antibiotic uptake (Charteris *et al*., 2001). However, *aacA-aphD* as well as *aadE* and *aphA3*, encoding aminoglycoside modifying enzymes, have previously been found in *L. acidophilus* and *L. salivarius* strains and in *L. curvatus* (Tenorio *et al*., 2001; Danielsen et al., 2005).

A possible explanation for the resistance of *Lactobacillus* to vancomycin may be due to the presence of D-Ala-D-Lac as the normal dipeptide in their peptidoglycan instead of the dipeptide D-alanine-D-alanine, the target for vancomycin activity (Handwerger *et al*., 1994; Klein *et al*., 2000). However, the resistance towards vancomycin has been demonstrated being as intrinsic and should not be compared with transmissible, plasmid-mediated resistance found in enterococci (Leclerc *et al.*, 1992).

The lactobacilli in the present study comprised strains resistant to tetracycline, chloramphenicol, and clindamycin. Tetracyclines are broad-spectrum antibiotics, with activity against Gram-positive and Gram-negative bacteria, *Chlamydia* spp., *Mycoplasma* spp., *Rickettsiae* spp. and some protozoa. Tetracyclines are the most commonly prescribed antibiotics within human medicine after beta-lactams, and are also widely used in veterinary medicine. Furthermore, tetracyclines have been used in horticulture, and are still used as growth promoters in some countries (Swedres, 2008). In bacteria, there are two ribosome-related mechanisms of tetracycline resistance, both of which are linked to the primary binding site of the drug on the ribosome. This resistance is caused by ribosomal protection proteins (RPPs) (Chopra and Roberts, 2001) or, more rarely, by mutations at nucleotide 1058 of the 16S rRNA (Ross *et al*., 1998). Tetracycline resistance genes tet (*K, L, M, O, Q, S, W*, 36) have been reported in various *Lactobacillus* species (Chopra and Roberts, 2001; Roberts, 2005; Ammor *et al.*, 2007). The most frequently found *tet* gene, tet(M), has previously been identified in strains of *L. plantarum, L. alimentarius, L. curvatus, L. casei, L. acidophilus, L. gasseri, L. crispatus* and *L. sakei* (Gevers *et al*., 2000; Klare *et al*., 2007), and also in *L. reuteri* (Van Hoek *et al*., 2008). Resistance to tetracycline is also commonly associated with efflux proteins. There are 26 *tet* efflux genes described so far, all of which confer resistance to tetracycline and doxycycline. Two of these genes, *tet*(K) and *tet*(L), are primarily found in Gram-positive bacteria. Efflux genes and genes encoding RPPs are both commonly associated with mobile genetic elements (Roberts, 2005).

Plasmid-encoded genes mediating chloramphenicol resistance (cat) have been identified in *L. plantarum, L. reuteri* and in *L. johnsonii*. The gene of the *L. plantarum* strain was similar to a streptococcal *cat* gene and could be transferred by conjugation with a helper plasmid to a Carnobacterium strain (Ahn *et al.*, 1992; Lin *et al.*, 1996; van Hoek *et al.*, 2008). Chloramphenicol is used for certain life-threatening infections such as typhoid fever, but in humans, it can cause fatal aplastic anaemia at therapeutic doses, limiting its use within human medicine. However,

chloramphenicol is used for several disease conditions in domestic animals, which could be a plausible explanation for the occurrence of the chloramphenicol resistant *L. reuteri* strain isolated from dog (Schwarz *et al*., 2004). Atypical resistance patterns among lactobacilli were recorded for the antibiotic acting as DNA synthesis inhibitor (Metronidazol). Since lactobacilli (lactic acid bacteria in general) have no hydrogenase activity (Church *et al*., 1996), resistance (MIC $\geq$ 32 µg/mL) to antibiotic may be obtained. In opposite to this, 23 isolates were susceptible. It seems that MRS components interfere with antibacterial activity of this antibiotic (Klare *et al*., 2007).

In fact, gene transfer is essential for bacteria to survive and adapt to new environments (Kurland *et al*., 2003). Strains intended for the use in food systems as starters or probiotics should therefore be carefully examined for antimicrobial susceptibility (Teuber *et al.*, 1999). However, there is still a lack of agreement on the resistance-susceptibility breakpoints for most antimicrobials in LAB. Distinguishing between intrinsic, nonspecific, and acquired resistance is difficult and requires that the antimicrobial-resistance patterns of many LAB species from different sources be compared. Besides the traditional clinical breakpoint, which may help clinicians in the choice of antibiotics, the term microbiological breakpoint has recently been defined. Microbiological breakpoints are set by studying the distribution of MICs in bacterial populations and the part of the population that clearly deviates from a susceptible majority is considered resistant (Olsson-Liljequist *et al.*, 1997). Microbiological breakpoints are thought to be more relevant than clinical breakpoints for the purpose of identifying bacterial strains with acquired and potentially transferable antibiotic resistances. Efforts should, therefore, be made to examine a large number of strains from different origins using the same methodology to clearly define such breakpoints for LAB.

Chapter 09

In vitro Inhibition of *Helicobacter pylori*

9. Susceptibility of *Helicobacter pylori* to Bac-F15, a bacteriocin produced by *Lactobacillus fermentum* BH1509.

9.1 INTRODUCTION

The use of functional starter cultures, a novel generation of starter cultures that offers functionalities beyond technological properties, is being explored. For instance, lactic acid bacteria (LAB) are capable of inhibiting various microorganisms in a food environment and display crucial antimicrobial properties with respect to food preservation and safety (Oppegård *et al*., 2007). In addition, it has been shown that some strains of LAB possess interesting health-promoting properties; one of the characteristics of these probiotics is the potential to combat gastrointestinal pathogenic bacteria such as *Escherichia coli, Salmonella* and *Helicobacter pylori* (De Vuyst and Leroy, 2007).

Helicobacter pylori infection is the major cause of chronic gastritis and peptic ulcer disease and is a risk factor for gastric cancer in humans. This infection is extremely common throughout the world, and its prevalence increases with age and lower socioeconomic status. Combinations of several drugs are now widely used for the eradication of *H. pylori*. Numerous clinical trials have indicated that eradication of *H. pylori* by treatment that includes bismuth or antisecretory drugs combined with antibiotics leads to healing of gastritis and drastically decrease the rate of peptic ulcer relapse. However, a limited number of antibiotics can be used, and drug resistance jeopardizes the success of treatment. Therefore, a search for new antimicrobial agents is warranted (Pinchuk *et al*., 2001). Several authors have previously reported that certain probiotic bacteria, such as *Lactobacillus* spp., exhibit inhibitory activity against *H. pylori in vitro* and *in vivo*. Various studies regarding the antagonistic effect of certain LAB strains, i.e., *Lactobacillus acidophilus*, *Lb. rhamnosus* GG, *Lb. salivarius*, and *Lb. gasseri*, against *Helicobacter pylori* have been reported (Midolo *et al*., 1995; Coconnier *et al*., 1998; Canducci *et al*., 2000; Armuzzi *et al*., 2001; Sakamoto *et al*., 2001). The anti-*H. pylori* effect is reported to organic acid production, competitive inhibition for the binding sites of mucous cells, and immunomodulation. However, the synthesis of bacteriocin-like compounds has rarely been associated with these antagonistic effects. The potential uses of bacteriocinogenic *Lb. fermentum* to combat *H. pylori* infections, including its responsible mechanisms, are not addressed and the characterization of the antimicrobial metabolites other than organic acids remains largely unexplored.

The aim of this study was to determine the susceptibility of of *H. pylori* to *Lb. fermentum* BH1509, a strain isolated from pollen, and to its supernatant as well as to characterize the antimicrobial protein secreted this bacterium. The final purpose is to find a possible alternative to antibiotic treatments for *H. pylori* strains. The possibility that isolated bacteriocin produced by *Lb. fermentum* could have the potential to inhibit the growth of *H. pylori* as well as some characteristics of the secreted protein is discussed.

9.2 MATERIALS AND METHODS

9.2.1 *Microorganisms and growth conditions*

Frozen *cultures of Lactobacillus fermentum* BH1509 (stored in MRS broth (Fluka) added with 20% glycerol) were grown in 5 mL of MRS broth supplemented with 0.05 % cysteine hydrochloride (Sigma) for 12–14 h at 37°C, sub-cultured twice in the same medium under the same conditions, and then cells were separated by centrifugation at 3000 *g* for 30 min. The inoculum for the cultures was 2%. The pellet was re-suspended in 5 mL of MRS broth and stored at –20 °C and the supernatants were separated for detection of bacteriocins.

H. pylori ATCC 43504 (a reference strain) and *H. pylori* GT1302 (an isolate provided by Dr. Bassem J., Laboratoire be Biotéchnologie de S'fax, Tunisie) were regenerated from stock cultures in brain heart infusion (BHI) broth containing 0.25% yeast extract, and 10% horse serum. *Helicobacter* culture was incubated in a gas jar with a micro-aerophilic atmosphere (gas-generating kit, BBL GasPack) at 37°C, and stored in the same medium supplemented with 20 % glycerol at –20°C. They were sub-cultured twice for 12–14 h incubated at 37°C micro-aerobically prior to perform the inhibition studies.

9.2.2 *Bacteriocin activity assay*

Samples of SCS in MRS broth were obtained by centrifugation (4000 *g*, 10 min, 4°C) of the 48 h BH1509 culture, followed by filtration of the supernatants through sterile filter of 0.45 µm pore size (Millipore, Bedford, MA, USA). The filtered supernatants were stored at −20 °C until assayed. The agar diffusion method described by Lin *et al*. (2011) was used. *Helicobacter pylori* strains (ATCC 43504 and GT1302) were cultured in Brucella broth containing 50 mL L^{-1} heat inactivated foetal bovine serum (FBS) for 48 h and then diluted to 10^8 colony-forming units (CFU) mL^{-1} with sterile phosphate-buffered saline (PBS; pH 7.2). Aliquots of 100 µL of the bacterial dilution were spread on the Brucella agar plates, and wells (6 mm in diameter) were made in the agar with a sterile glass rod. Aliquots of 70 µL of the following solutions; whole bacterial culture without neutralization (acidic, pH 6.5), neutralised culture of BH1509, BH1509-SCS without neutralization (pH 6.5), neutralized BH1509-SCS (adjusted to pH 7 with 1.0 mol L^{-1} NaOH), BH1509-SCS heat treated at 100 °C for 10 min, BH1509-SCS digested with 0.5 mg mL^{-1} proteinase K (Sigma, St. Louis, MO, USA) at 25 °C for 30 min, and MRS broth (pH 7.2) as the control; were dropped into the wells. Up to 50 mM N-acetyl cysteine (NAC; Sigma) was added into the well containing native BH1509-SCS. The plates were incubated at 37 °C for 72 h under microaerophilic conditions. The diameters of the inhibition zones around the wells were then measured with callipers. Results are expressed as the mean diameter of triplicate independent experiments.

9.2.3 *Growth and bacteriocin production kinetics*

The strain BH1509 was regenerated from stock cultures by sub-culturing three times in MRS broth incubated at 37°C during 18 h. Thereafter, ten milliliters of the overnight strain culture was inoculated into 300 mL of MRS broth and incubated at 37 °C. At 2-h intervals, 1 ml samples were removed and the optical density (OD. λ=590 nm) was determined. Mean-while 10 mL culture was removed to prepare SCS (pH 6.5). The antibacterial activities of the SCSs were determined by well diffusion assay previously described. *Helicobacter pylori* GT1302 was used as the indicator strain. Medium pH and bacteriocin titer were also recorded.

9.2.4 *Mode of action*

H. pylori GT1302 was used for the study of mode of the bacteriocin action. The bacterium was inoculated in the same broth to obtain final turbidity equal to an OD. (λ=650 nm) 0.2 and incubated at 37°C for 72 h under microaerophilic conditions. Six hundred AU/mL of GT1302 was added at various growth stage of indicator cell and incubated at 37 °C in a shaker rotating at 115 rpm for 72 h. The samples were taken at different time intervals to measure absorbance at 650 nm.

9.2.5 *Antagonistic spectrum of BH1509 supernatant*

To determine the antimicrobial spectrum of the crude bacteriocin sample, well diffusion assay was used. Soft BHI agar (0.7%) was seeded (1% v/v) with the indicator bacteria (Table 29) and poured in Petri dishes containing approximately 18 mL BHI agar (1.5 % agar). Wells (7 mm) were created and filled with 70 µL of BH1509-SCS obtained from an overnight culture. Instead of seeding the soft agar with as for bacteria, 50 µL of a fungal solution were inoculated on the surface of soft BHI agar before wells are made. The plates were incubated at the appropriate growth conditions, and inhibitory effect was observed.

9.2.6 *Partial purification of the bacteriocin*

a. Separation of Bacteriocin by ammonium sulfate precipitation

The SCS was obtained by centrifuging the culture medium (1000 mL) at 8,500 x g and 4 °C for 15 min, and neutralized to pH 8.0 with 1 N sodium hydroxide. The supernatant was subjected to ultrafiltration (Sigma chemicals, St. Louis, MO) using a 10 kDa cutoff membrane cartridge filter. Ammonium sulfate was slowly added to the resulting filtrate to produce 80% saturation and stirred overnight at 4°C. The precipitated proteins were collected by centrifugation at 10,000 *g* for 20 min at 4 °C and re-suspended in a minimal quantity of a 10 mM ammonium acetate buffer (pH 6.0). The suspension was then dialyzed overnight at 4°C against the same buffer with a 1 kDa cutoff (Sigma chemicals, St. Louis, MO). The dialysate was used as the crude sample.

b. Sephadex G-50 Chromatography

The dialyzed sample was applied to a Sephadex G-50 gel filtration column (35 x 2 cm, Pharmacia Biotechnology, Uppsala, Sweden) connected to an Akta Prime plus protein purification system (GE Healthcare, Uppsala, Sweden). The gel column was equilibrated with 10 mM ammonium acetate buffer (pH 6.0), After washing with 1000 ml of the same buffer, the absorbed sample was eluted using 10 mM ammonium acetate buffer (pH 6.0) with 0 to 0.5 M NaCl at a flow rate of 0.5 mL/min and fraction size of 5.0 ml. For each fraction, a bacteriocin activity assay was performed. The active fraction was concentrated by lyophilization, and fractions of 5 ml were collected sequentially for protein content as illustrated by Bradford (1976) and activity determination. The active fractions were recovered and dialyzed against the ammonium acetate buffer as described previously. The obtained dialysate was used as the partially purified sample.

c. Determination of approximate molecular mass and bioautographic Assay

Active fractions eluted from the Sephadex G-50 column were analyzed by SDS-PAGE. Electrophoresis was performed using an 18% polyacrylamide gel at a constant voltage of 120 V for 3.5 h. The gel was fixed in 40% (v/v) methanol/l0% glacial acetic acid for 2 h. Half of the gel was then stained with Coomassie brilliant blue R-250 (Bio BASIC Inc.) A low molecular mass protein marker with sizes ranging from 4.1 - 66 kDa was used. The other half of the gel was washed 2 times for 4 h in distilled water with gentle agitation to remove the SDS. The gel was placed in a petri dish containing a 2% agar medium and then overlaid with a pre-cooled 0.7% agar medium seeded with 1.5% (v/v) of the indicator organism (*H. pylori* GT1302) (Jiang *et al*., 2012). The plate was incubated under microaerophilic conditions at 37°C for three days.

9.2.7 *Effect of pH and temperature*

A crud bacteriocin sample Bac-F15 was resuspended in different buffer solutions at 50 mM, ranging from pH 2 to 9 (citrate, for pH 2 - 6; phosphate for pH 7; Tris-HCl for pH 8 - 9), and incubated for 24 h at 4 °C. The antimicrobial activity of Bac-F15 was expressed in terms of inhibition diameter zone (mm) and compared with the untreated controls. The thermal stability of Bac-F15 was monitored at different temperatures. Accordingly, Bac-F15 was resuspended in 25 mM Tris-HCl buffer at pH 8, subjected to different temperatures (50, 70, 80, 90, and 100 °C for 10 min) prior to performing activity assays. *H. pylori* GT1302. was used as indicator bacterium.

9.2.8 *Stabilities of purified Bac-F15 bacteriocin*

The effect of detergent on bacteriocin activity and stability was investigated. This involved the incubation of pure Bac-F15 in the presence of a broad range of detergent [Tween 40, Tween 60, Triton X-100, SDS (sodium dodecyl sulphate), and urea] at final concentrations of (1 mg/mL). In this case the bacteriocin Bac-F15, which was

resuspended in buffer A and without any denaturing agents, was used as the control. Each sample and control was incubated at 37 °C for 24 h. The activity was assayed as described above. To determine the sensitivity of a number of enzymes on active Bac-F15, a variety of enzymes were added at a final concentration of 1 mg/mL. All enzymes were dissolved in buffers as recommended by the supplier. Enzymes used were: lipase, catalase, trypsin, pronase E, and proteinase K. Following 24 h of incubation at 37 °C, enzymes were heat inactivated (70°C at 10 min.) and tested for inhibition against indicator bacterium *H. pylori* GT1302. Untreated bacteriocin plus buffers, buffers alone were used as controls.

9.3 RESULTS

9.3.1 *Susceptibility of H. pylori by Agar well diffusion assay*

Agar well diffusion assay realized on two *H. pylori* trains as indicator bacteria indicate that the *Lb. fermentum* BH1509 produces a molecule inhibiting the pathogenic bacteria. After overnight growth of *Lb. fermentum* BH1509, the medium pH does not change greatly (final pH 6.5) (Figure 17). However, there was no difference between the activity of whole bacterial culture and its supernatant as well as between acidic or neutralized supernatant eliminating therefore the plausible action of organic acids. Clear inhibition of the pathogen by heat treated-SCS (100°C/10min) indicates the heat stability of the active molecule (s). In addition the active molecule was sensitive to proteolytic treatment by proteinase K. From another point of view, there was no difference between the antimicrobial activities against the two *H. pylori* strains.

9.3.2 *Growth and bacteriocin production kinetics*

As reported above, during 24 h of incubation, the pH of the medium reache 6.5 compared to the initial pH (7.0). The exponential phase of growth started at after 4 hours, followed by the stationary phase after 12 hours of incubation. Bacteriocin production was observed after 8 hours and reached its maximum level after other 8 hours of incubation (Figure 18). This maximum concentration (9600 AU mL^{-1}) persists two hours (during the beginning of the stationary phase, followed by decrease during the rest of the stationary phase (Figure 18).

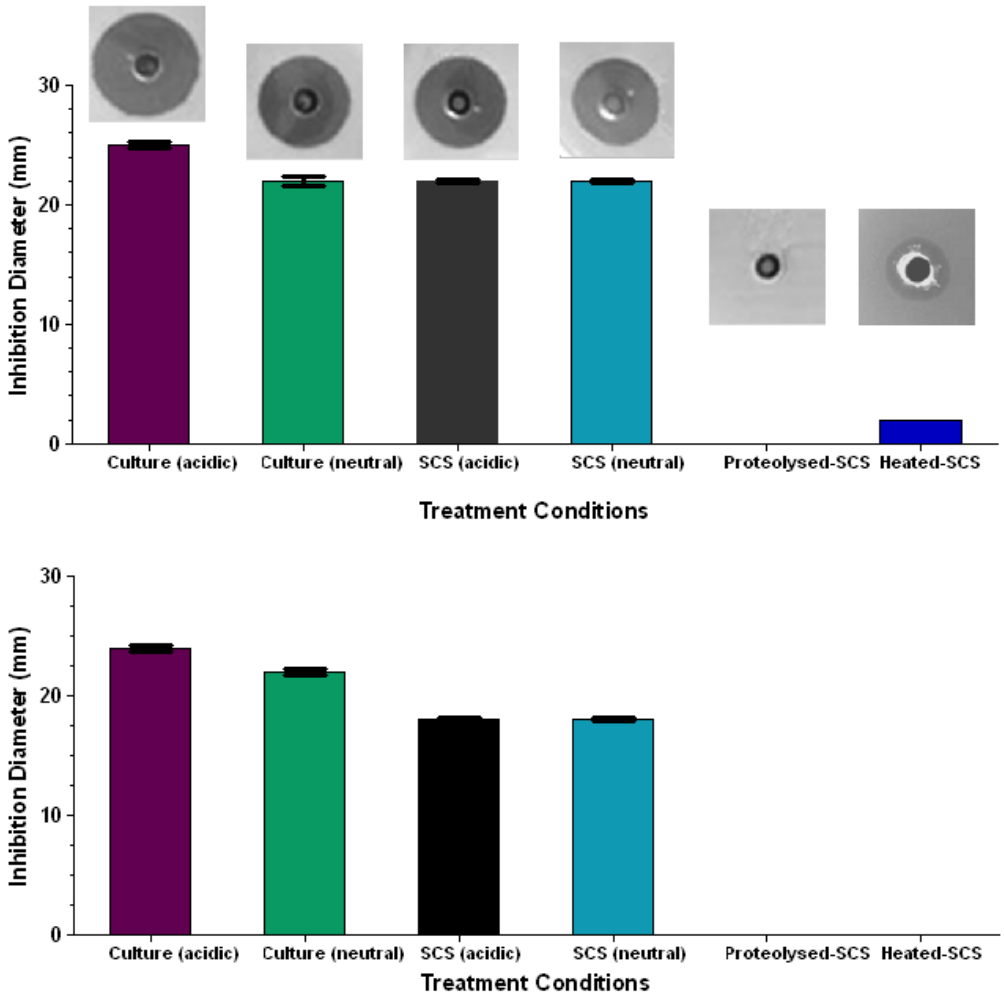


Figure 17: Inhibition of *H. pylori* strain GT1302 (A) and strain ATCC 43504 (B) by different solutions using well diffusion assay. Whole bacterial culture of *Lactobacillus fermentum* BH1509; pH adjusted (7.0) of whole bacterial culture; acidic spent culture supernatant (SCS); neutralized-SCS; bacteria-SCS treated with 0.5 mg mL^{-1} Proteinase K; and thermal treated-SCS (100°C/10 min).

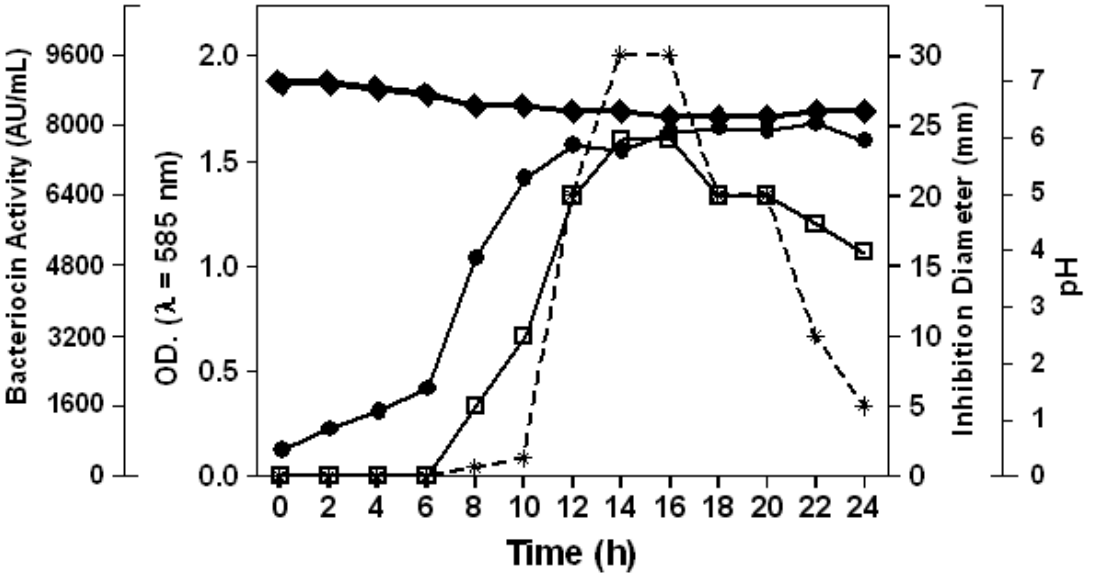


Figure 18: Bacterial growth, bacteriocin production, and medium pH changes. The growth curve analysis of strain *Lactobacillus fermentum* BH1509 indicates that the production of bacteriocin initiated at begaining of logarithmic phase and reaches its maximum concentration at the stationary phase. Bacterial growth (expressed as absorption at 585 nm) is indicated by circles (-●-, right y axes), while the inhibition diameter by opened squares (-□-, left y axes). Medium pH variation is indicated by filled sqauars (-■-, left y axes). Bacteriocin activity (AU/mL) is indicated Discrete line (---, right y axes).

9.3.3 *Antagonistic spectrum of BH1509 supernatant*

The antibacterial spectrum of the bacterial supernatant is shown in Table 29. The supernatant inhibited Gram-positive bacteria *Micrococcus luteus*, *Staphylococcus aureus*, and *Listeria innocua*. In addition, significant activity against Gram negative ones; *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia, Helicobacter pylori*, and *Campylobacter jejuni* was observed. However, no activity was detected towards two tested fungi, *Candida tropicalis* R2 CIP203 and *Aspergillus parasiticus.* These results demonstrate the broad antibacterial activity generated by the strain BH1509 in the supernatant.

Table 29: Antagonistic spectrum of Bac-F15. The inhibitory level was estimated by measuring the diameter of the inhibition zone of the indicator strain.

Indicator strains		Diameter of inhibition zone (mm)
Gram-positive bacteria	*Micrococcus luteus* LB 14110	15 ± 1.5
	Staphylococcus aureus ATCC 6538	22 ± 2.0
	Listeria ivanovii BUG 496	20 ± 2.0
	Enterococcus faecalis JH 2-2	25 ± 2.0
Gram-negative bacteria	*Escherichia coli* ATCC 8739	13 ± 1.1
	Salmonella typhimurium	20 ± 2.0
	Stenotrophomonas maltophilia	19 ± 1.8
	Pseudomonas aeruginosa ATCC 49189	27 ± 3.0
	Helicobacter pylori GT1302	25 ± 2.0
	Helicobacter pylori ATCC 43504	24 ± 2.0
	Campylobacter jejuni GT404	22 ± 2.0
Fungi	*Candida tropicalis* R2 CIP203	-
	Aspergillus parasiticus	-

9.3.4 *Effect of pH, temperature, surfactants and enzymes on Bac-F15*

The purified bacteriocin, Bac-F15, was found to be resistant to acidity and moderate alkalinity. It retains its total activity at pHs ranging from 4 to 8. At pH 2 and 3, the residual activity was 37 and 53% respectively. Under alkaline conditions (pH 9), only 13% of the total activity was recorded (Table 30). Bac-F15 was found to be heat resistant till the temperature of 100°C for 10 min. At temperatures of 50°C, 70°C, 80°C 90°C and 100°C for 10 min 100%, 76%, 48%, 29% and 17% activity was retained respectively (Table 30). It was resistant to Tween 40, Tween 60, Triton X-100, SDS, and Urea (Table 3). It was sensitive to, trypsin, protease, Proteinase K but insensitive to catalase, confirming that inhibition was due to proteinaceous molecule i.e. bacteriocin and not due to hydrogen peroxide. Its activity was not reduced by lipase indicating that there was no structural modification by lipid moiety (Table 30).

9.3.5 *Mode of action*

In order to investigate the mode of bacteriocin action, Bac-F15 was added to the liquid culture of *H. pylori* GT1302. The addition of bacteriocin (600 AU/ml) to the logarithmic phase of bacterial cells decreased the optical density at 650 nm at each time interval (Figure 19). When adding the bacteriocin at the beginning of the logarithmic growth phase, the optical density of the bacterial culture does not increase indicating a total bacterial growth inhibition. During the exponential phase, the optical density decreases rapidly after the addition of the bacteriocin compared to the control culture (Figure 19).

Table 30: Effect of pH, temperature, surfactants and enzymes on antibacterial activity of Bac-F15.

Factors	Treatment	Diameter (mm) of zone of inhibition
pH	pH 2.0	10.00 ± 0.45
	pH 3.0	12.30 ± 0.31
	pH 4.0	24.00 ± 0.26
	pH 5.0	24.00 ± 0.21
	pH 6.0	24.00 ± 0.32
	pH 7.0	24.00 ± 0.18
	pH 8.0	22.60 ± 0.34
	pH 9.0	05.30 ± 0.15
Temperature	50 for 10 min	24.00 ± 0.21
	70 for 10 min	18.20 ± 0.18
	80 °C for 10 min	15.00 ± 0.20
	90 °C for 10 min	13.00 ± 0.12
	100 °C for 10 min	08.00 ± 0.00
Surfactants	Tween 40 (1 mg/ml)	24.00 ± 0.09
	Tween 60 (1 mg/ml)	24.00 ± 0.19
	Triton X-100 (1 mg/ml)	23.85 ± 0.17
	SDS (1 mg/ml)	23.80 ± 0.09
	Urea (1 mg/ml)	23.80 ± 0.11
Enzymes	Lipase	23.90 ± 0.20
	Catalase	23.90 ± 0.14
	Trypsin	-
	Pronase E	-
	Proteinase K	-

–, no inhibition zone

Bacteriocin activity was determined against *H. pylori* GT1302

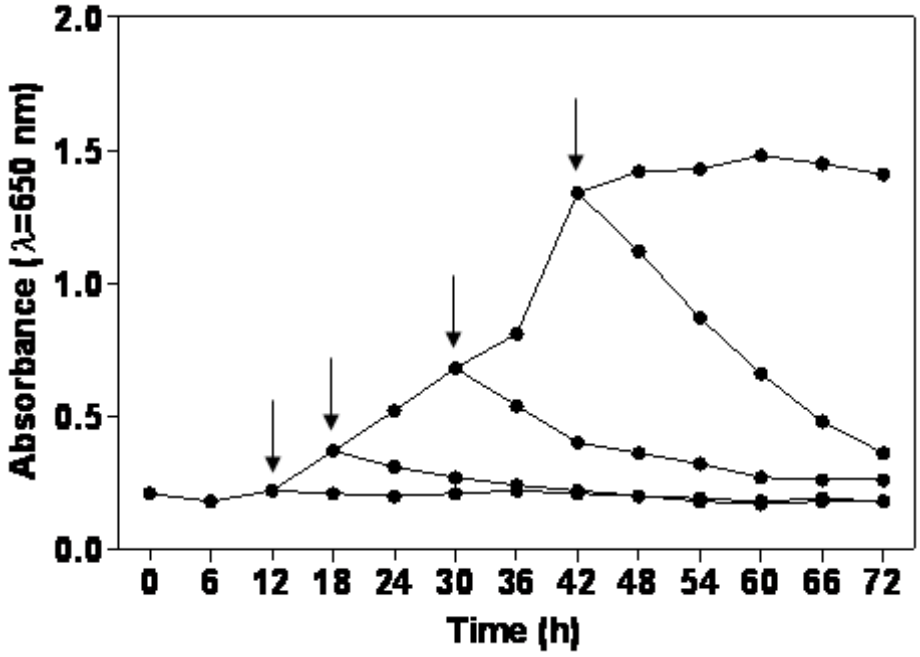


Figure 19: Effect of the bacteriocin of *Lactobacillus fermentum* BH1509 on the growth of *Helicobacter pylori* GT1302 at 37 °C. Arrows indicate addition of the bacteriocin (600 AU/ml).

9.3.6 *Partial purification of the bacteriocin*

Purification steps of the bacteriocin are summarized in Table 31. To purify Bac-F15, SCS was ultrafiltered through 10 kDa membrane and then ammonium sulphate precipitated, dialyzed and subjected to Sephadex G-50 gel chromatography. The bacteriocin activity of the supernatant was also 9600 AU/mL. The bacteriocin, Bac-F15, was passed through cellulose membrane with 10 kDa but could not through one with a 1 kDa cut-off. Purification by Sephadex G-50 chromatography, revealed nine peaks. Only one of the four eluted peaks showed antibacterial activity (Figure 20). The activity, yield and the purification fold of protein, along with the purification procedure, are summarized in Table 31. An active fraction from Sephadex G-50 chromatography was analyzed by SDS-PAGE, which yielded a single band which has a molecular weight below 6.5 kDa and superior to 4.1 KDa on the stained gel. Moreover, Clear areas appeared around the zones of migration of the protein during electrophoresis (Figure 21). This corresponds to the bacteriocin band obtained after purification.

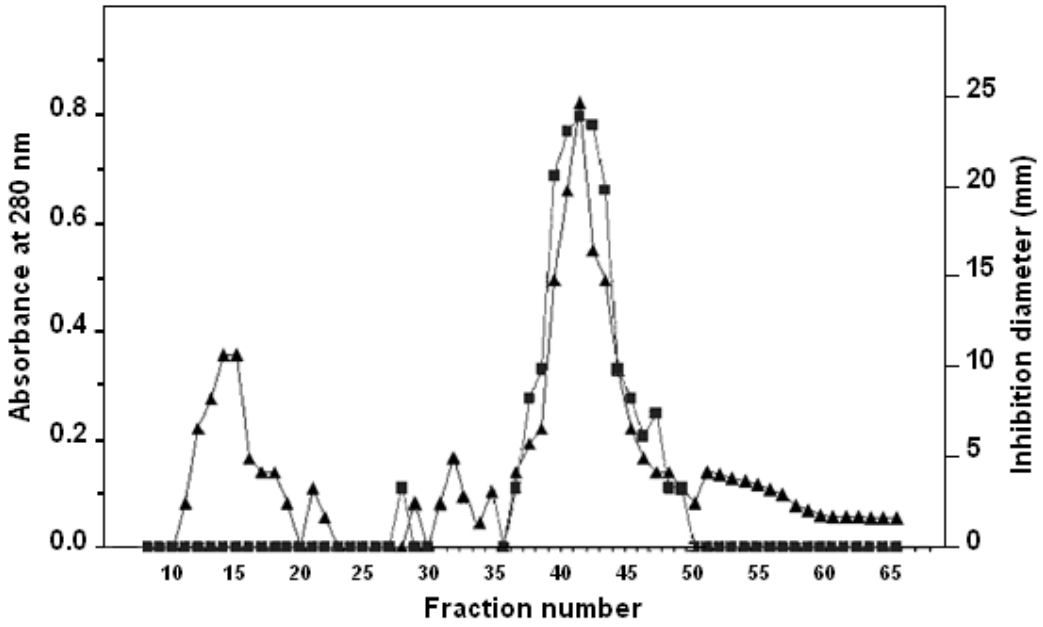


Figure 20: Elution profile and antimicrobial activity of collected fractions of the ammonium sulfate precipitated bacteriocin from *Lactobacillus fermentum* BH1509 on Sephadex G-50 column. Bioactivity was determined using *Helicobacter pylori* GT1302 as indicator strain.

Table 31: Purification of bacteriocin, Bac-F15, produced by *Lactobacillus fermentum* BH1509.

Step	**Volume (mL)**	**Bacteriocin activity (AU/mL)**	**Total activity (AU)[a]**	**Total protein (mg)**	**Specific activity (AU/mg)**	**Purification (fold)**	**Yield (%)**
Spent culture supernatant	1000	9600	$9.6x10^6$	20,3	473	1	100
Ammonium sulfate precipitation/ ultrfiltration	100	72000	$7.2x10^6$	4.056	17751.48	37.53	37.52
Sephadex G-50 gel filtration chromatography	28.2	57600	$1.62x10^6$	0.06	96000	203	5.41

[a] Antibacterial activity (in arbitrary units [AU]) was assayed by agar well diffusion assay using *Helicobacter pylori* GT1302 as an indicator strain.

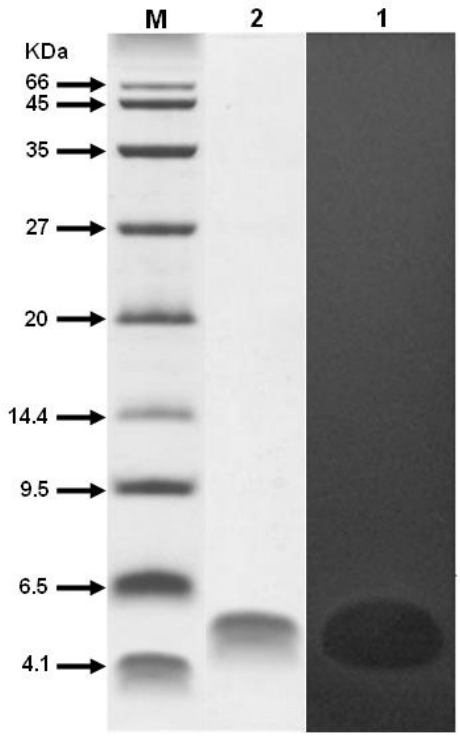


Figure 21: SDS-PAGE and antibacterial analyses of Bac-HB15. Lane M: low molecular weight markers; Lane 1: bacteriocin Bac-F15; Lane 2: gel overlaid with soft agar seeded with 1.5% (v/v) *H. Pylori* GT1302.

1

9.3.7 DISCUSSION

In this study the inhibition zone of BH1509-SCS against *H. pylori* was measured (Table 28 and Figure 17).

The results showed that the strain BH1509 had better anti-*Helicobacter* effect without acidification of the culture broth and more efficient bactericidal activity against both *H. pylori* strains according to the broth based inhibition assay (Figure 19). In addition, there were no obvious differences in bactericidal activity between whole culture BH15, BH15-SCS (acidic or neutralized). However, this activity against *H. pylori* was lost with protease-digested BH15-SCS (Table 1). These results suggest that the anti-*H. pylori* activity of SCS is not related to concentration of organic acids and the pH value but is associated with the protein components. Scars studies on the inhibition of *H. pylori* by *Lb. fermentum* and its bacteriocins are available in the literature (Kaur *et al.*, 2012; Kaur *et al.*, 2013). However, most studies on the anti-Helicobacter pylori effects of lactic acid bacteria focus on the inactivation of *H. pylori* by organic acids and hydrogen peroxide produced by LAB (Gotteland et al., 2006). In order to investigate the mode of action of Bac-F15, *H. pylori* cultures were subjected to the action of the bacteriocin during various stages of the exponential growth. The dramatic decrease in the optical density may be due to the leakage of intracellular components to the medium after cell lysis caused by bacteriocin treatment which indicates the bactericidal effect of the protein Bac-F15. However, further experimentation is needed to elucidate the mode of action of Bac-F15, including its effects on leakage of certain intracellular substances and on cell morphology.

The bacteriocin produced by *Lb. fermentum* BH1509 was sensitive to proteolytic enzymes, indicating that the inhibitory material was proteinaceous. Its antibacterial activity was not abolished by treatment with catalase. Lipase treatment of the protein reveals that the protein does not contain lipidic moiety in its structure. Like most of the known bacteriocins produced by *Lb. fermentum* strains, it is a heat-stable and low molecular mass (between 4.1 and 6.5 KDa) peptide. The bacteriocin activity was stable at a wide range of pH levels (4.0 to 8.0). According to the classification reported by Klaenhammer (1988), the bacteriocin produced by *Lb. fermentum* BH1509 belongs to the class II lactic acid bacteria bacteriocins.

The bacteriocin Bac-F15 produced by *Lb. fermentum* BH1509 showed a wide inhibitory spectrum. A noteworthy observation was the inhibition of the Gram positive as well as Gram negative bacteria; *Micrococcus luteus*, *Staphylococcus aureus*, and *Listeria innocua*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Helicobacter pylori*, and *Campylobacter jejuni*. Generally, bacteriocins from lactic acid bacteria are active only toward taxonomically closely related bacteria (Klaenhammer, 1988; 1993).

A wide inhibitory spectrum seems to be common among bacteriocin-producing isolates from the group III of the genus *Lactobacillus* (including *Lb. fermentum*). Our findings are consistent with studies reported elsewhere. For example, lactacin F from

Lb. johnsonii VPl 11088 displays bactericidal activity toward other lactobacilli and *Enterobacter faecalis* (Muriana and Klaenhammer, 1991). Acidocins J1129 and J 1132 (Tahara and Kanatani, 1997), and acidocins LF221 A and LF213 B (Bogovic-Matijasic *et al*., 1998), are active against closely related lactic acid bacteria. Many low molecular mass, proteinaceous, antibacterial compounds from *Lb. acidophilus* showing broad inhibitory spectra, including inhibition of Gram-positive and Gram-negative bacteria, have been classified as small, heat-stable, bacteriocin-like peptides (De Vuyst and Vandamme, 1994). In addition, Yan and Lee (1997) and Pascual *et al*. (2008) studied the antimicrobial activity of two bacteriocins froduced by *Lb. fermentum*. These bacteriocins exhibit wide activity spectra. They inhibit Gram positive and Gram negative bacteria. The bacteriocin, Bac-F15, was produced during the exponential growth phase of *Lb. fermentum* BH1509. The inhibitory activity against *H. pylori* reached a maximum (9600 AU/ml) at 14 h of culture. The bacteriocin activity remained stable when the cells entered the stationary growth phase (Figure 2). Several bacteriocins produced by lactic acid bacteria presented the same profile, noatbely fermentcin B (Yan and Lee1997).

In conclusion, in the present study, a new bacteriocin-producing strain, *Lb. fermentum* BH1509, was isolated from Algerian pollen. The inhibitory protein, bacteriocin Bac-F15, secreted by the bacterium inhibits *Helicobacter pylori*. Its molecular masse situated between 4100 and 6500 Da. The exact molecular weight and its amino acid structure remains to be determined. The bacteriocin, Bac-F15, is heat stable and exhibits a wide range of antagonistic potential against both Gram positive and Gram negative bacteria. It has a bactericidal mode of action upon *Helicobacter pylori* strains. Further studies on the biosynthesis of this bacteriocin and its biotechnological and therapeutic applications, notably for the bio-control of Helicobacter pylori infection should be addressed.

III.

General Discussion

GENERAL DISCUSSIONS AND CONCLUSIONS

This thesis aimed to characterize LAB found in pollen grains and to understand some probiotic traits of selected strains (especially belonging to the genus *Lactobacillus*) with respect to the inhibition of *Helicobacter pylori*. Preliminary investigations (Chapter 3, 4 and 5) address the microbial content as well as dynamics of some LAB genera in pollen samples. By focusing on antagonistic isolated strains and phenotypic based numerical clustering method, Chapter 6 provides in depth a face view about different LAB genera and species that can be found and contribute to the microbial flora of pollen. In Chapter 7, probiotic characteristics of selected *Lactobacillus* strains were investigated. It is relevant that *Lb. plantarum* and *Lb. fermentum* were more tolerant strains compared to the other studied isolates. Based on 16S rDNA gene sequencing, 20 strains belonging to *Lactobacillus* were characterized. In Chapter 8, antibiotic susceptibility of isolated lactobacilli reveals a resistance profile to some antibiotics active against Gram-negative and Gram-positive bacteria. Given its potential as anti-*Helicobacter pylori*, *Lb. fermentum* BH1509, a strain resistant to acid and bile salts, produces a low molecular weight bacteriocin. The bacterium and its supernatant spent culture inhibit the growth of *H. pylori* (Chapter 9).

As discussed in Chapters 3 and 4, microbial loads of analyzed samples reveal clearly that produced pollens are very contaminated and is considered as a potent carrier for pathogenic bacteria and fungi. However, from quality control point of view, the presence of enterobacteria in foods is an indication of recent fecal contamination. As there is not a national legislation for the production and processing of pollen, microbiological guidelines for these purposes are of great importance. According to Campos *et al*. (2008), pollens should have the following microbial aspects: absence of *Salmonella*/10g; absence of *Staphylococcus* and *Escherichia coli* /01 g; TAPC coold not exceed than 10^5 CFU /g; TMYC should be less than 5. 10^4 CFU/g; and the maximum of enterobacteria is 100 CFU/g. Therefore, understanding the microbial loads of pollen before consumption should be taken in consideration. The high incidence of fungi in pollen grains studied in this work point out that raw pollen in not ready-to-eat food without processing (drying). In Algeria, pollen is dried naturally by exposing it to sunlight or oven heated at a low temperature (less than 45°C). Serra and Alegret (1986) recommend the avoidance of natural pollen drying, because at low temperatures, fungal growth and mycotoxin production might occur. If raw pollen was stored without processing, fungi can flourish under appropriate conditions (humidity and temperature) as pollen is a suitable plant product either for fungal growth or mycotoxin production. However, mycotoxins production, such as ochratoxin A (OTA) and aflatoxins B_1, B_2, G_1, and G_2 by pollen associated fungi is known (Gonzalez *et al*., 2005; Pitta and Markaki, 2010). Aflatoxins and ochratoxin A are thermostable and carcinogenic molecules

(Boudra *et al*., 1995; Pitta and Markaki, 2010), thus, pollen dried naturally or not may present a threat for human and animal health. From another point of view, the slightly acidic aspect of the analyzed pollen samples favors the development of fungi and mycotoxin production. *Aspergillus, Penicillium, Alternaria, Mucor* and others were recovered from pollen. They are involved in various diseases such as allergic illness, mycotoxicosis, and aflatoxicosis (Martin, 1974). In addition, the recovery of potent pathogenic bacteria belonging to the family *Enterobacteriaceae* is another proof of unsatisfactory and unacceptable pollens set for human consumption in local markets. A greatly intension should be taken developing legislation considering pollens and pollen extracts as well as standards for microbial quality control of this type of products. More importantly, expanded studies about pollen processing and preservation to reduce microbial load and pathogen elimination are necessary.

The LAB group is a recognised source of bacteria for use in the food-industry and as probiotic supplements. Accordingly, this bacterial group used for these purposes have been rigorously characterised.

LAB are ubiquitous in nature. They were isolated from various foods such as milk, meat, cereals and vegetables (McKay and Baldwin, 1990). As pointed in Chapter 5, LAB grew in laboratory fermented pollen. Growth succession of major LAB genera occurs with time and *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc* were recovered. The plants from which pollens were collected and honeybees were the main sources of LAB. Several studies were carried out on the distribution of these bacteria in plants and plant products, but for the best of our knowledge, there were no reports about their occurrence in pollens. Presumably the non abundance of these bacteria in this type of products results from their inhibition by some pollen products.

The antimicrobial compounds produced by LAB can inhibit the growth of pathogenic bacteria like members of *Entarobactariaceae* and *Helicobacter pylori* (Raccah *et al*. 1979, Smith and Palumbo 1983, Cintas *et al*. 1998). There have been many reports on the bacteriocins produced by LAB. These bacteriocins are of a proteinaceous nature (Klaenhammer 1993).

Based on selective LAB isolation, five hundred and sixty seven isolates of lactic acid bacteria were recovered from row bee-pollen grains as stated in Chapter 6. The 54 LAB effective strains were characterized by means of phenotypic tests. The relationships among the phenotypically characterized strains of lactic acid bacteria were determined by cluster analysis.

Most of the active isolates were assigned to *Lactobacillus*, especially *Lb. plantarum* and *Pediococcus pentosaceus* and *Pediococcus acidilactici*. Most commonly, strains of *Pediococcus acidilactici* and *P. pentosaceus* have been reported to produce bacteriocins (Anastasiadou *et al*., 2008). Pediocins form a group of bacteriocins belonging to the class IIa of bacteriocins, characterized as ‘‘antilisterial” (Papagianni, 2003). They inhibit several gram-positive spoilage and pathogenic bacteria. The spectra of antimicrobial activity of pediocins produced by strains of *P. acidilactici*

and *P. pentosaceus* have been found to be similar and this has been attributed to the phylogenetically close relation of the producer organisms (Collins *et al*., 1991). Based on phenotypic profiling, fifty four bacteriocin-like producing isolates of lactic acid bacteria were characterized from different raw pollen samples. The Simple Matching Coefficient (S_{SM}) cluster analysis resulted in five major clusters (A, C, E, F, and I, containing three or more strains), defined at the 79.00 % similarity level (Figure | 03). The following four other minor clusters were defined at the 79 % S_{SM} similarity level, (B, D, G, and H, two two-member cluster and two one-member clusters). As hypothesized by Siezen *et al*., (2010), the fermentative profile reflects the original habitat and lactose utilization is less prevalent in plant isolates with respect to those from cheese and human gastro-intestinal tract. Indeed, lactose is fermented by most of the isolates, except isolates of *Pediococcus* from clusters I, E and isolates of *Lactobacillus* sp. from cluster A. The inability of plant related LAB to ferment lactose was presumably due to the relatively recent acquisitions, via horizontal gene transfer and subsequent natural selection, of lactose metabolic genes, which are often plasmid encoded in dairy and human strains (Siezen *et al*., 2005). In opposite to the findings of Cagno *et al*. (2010) who studied *Lactobacillus plantarum* from vegetables and fruits, all *Lactobacillus plantarum* isolates of this study use this carbon source. However, based on the limited used tests, phenotypic profiles did not cluster the isolates according to the original habitat. This was probably because isolates from raw materials having a very similar chemical composition were compared. Or pollen residing lactic acid bacteria were from animal as well as plant origin. Nevertheless, phenotypic profiling was useful to understand the manifestations of environmental adaptation which will be reflected on the technological processes.

Phenotypic characterization was followed by 16S rRNA gene sequencing of 20 strains using universal amplification primers (Chapter 6 and 7). Several strains were not assigned to their exact taxonomic status by this method. Therefore, other molecular based characterization approaches should be used. It is known that this method is not efficient for the discrimination between *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus.* They are genotypically closely related and show highly similar phenotypes. As reported elsewhere (Rodas *et al*. 2003), physiological and biochemical criteria used for LAB strain identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, a clear identification to species level by simple phenotypic tests may be troublesome and inaccurate. Molecular methods used for discrimination of LAB strains to genus and species level are more efficient than phenotypic approaches.

Selected ten *Lactobacillus* strains were subjected for acid and bile salts challenges in Chapter 8. Remarkable acid tolerance and survivability rates were obtained in these experiments using *lactobacillus* strains from pollen (a bee hive product). This

tolerance may be due to adaptation of the strains to the known acidity of pollen grains. In the present results, lower pH values (1.5 and 2.0) affect greatly bacterial cell viability. One explanation that this might be due to the fact that at a pH lower than the pKa value of organic acids (derived from food and from gut), a major portion of the acid is in the non-ionized form, which is permeable through the cell membrane of microbes. Therefore, at lower pH there is a constant influx of organic acids into the cells which ultimately results in death. In addition, the ten *lactobacillus* strains were selected to test their ability to survive in simulated small intestinal juice with three different bile concentrations (0.3, 0.5, and 1.0%). It was considered that 0.45% of bile is sufficient to determine any resistant strains (Buntin *et al*., 2008). As shown in Table 4, 50% of strains survived simulated small intestinal juice with 1.0% bile salt for ten hours with percent cell viablity more than 50%. Tow strains (BH1507 and BH1509) were observed to be the most bile tolerant with survival rates of 76.4 and 77.5 %, respectively. According to Serrazanetti *et al*. (2009), the small intestinal juice tolerance of probiotic bacteria was strain dependent. Bile resistance of *Lactobacillus* spp. is related to the specific enzyme activity of bile salt hydrolase (BSH) which helps the hydrolysis of conjugated bile and thus reduces its toxic effects (Du Toit *et al*., 1998; Erkkila and Petaja, 2000), which are then readily excreted from the GI-tract (Maragkoudakis *et al.*, 2006). This particular enzyme decreases bile solubility and thus weakening its detergent effect.

In Chapter 9, the results showed that *Lactobacillus fermentum* BH1509 had better anti-*Helicobacter* effect without acidification of the culture broth and more efficient bactericidal activity against both *H. pylori* strains according to the broth based inhibition assay. In addition, there were no obvious differences in bactericidal activity between whole culture BH15, BH15-SCS (acidic or neutralized). This strain produces a low molecular weight bacteriocin, Bac-F15 (between 4.1 and 6.5 KDa). Generally, bacteriocins from lactic acid bacteria are active only toward taxonomically closely related bacteria (Klaenhammer, 1988; 1993). The protein secreted by BH1509 showed a wide inhibitory spectrum. A noteworthy observation was the inhibition of the Gram positive as well as Gram negative bacteria; *Micrococcus luteus*, *Staphylococcus aureus*, and *Listeria innocua*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Helicobacter pylori*, and *Campylobacter jejuni*. In addition, Pascual *et al*. (2008) and Yan and Lee (1997) studied the antimicrobial activity of two bacteriocins froduced by *Lb. fermentum*. These bacteriocins exhibit wide activity spectra. They inhibit Gram positive and Gram negative bacteria. The bacteriocin, Bac-F15, was produced during the exponential growth phase of *Lb. fermentum* BH1509. The inhibitory activity against *H. pylori* reached a maximum (9600 AU/ml) at 14 h of culture. The bacteriocin activity remained stable when the cells entered the stationary growth phase (Figure 2). Several bacteriocins produced by lactic acid bacteria presented the same profile, noatbely fermentcin B (Yan and Lee 1997).

In this study, we have confirmed that pollens available for consumption are contaminated and harbor a set of undesirable microorganisms (bacteria and fungi). Microbiological quality control tests indicate the presence of potentially pathogenic bacteria (*Salmonella*, *Shigella*, *E. coli*) and mycotoxigenic molds (*Aspergillus flavus*, *Aspergillus niger*) and pointed out that this beehive product was marketed without any safety regulation. It is a hazardous food material.

From another aspect, pollens are a suitable carrier for LAB. In the current study, 54 bacteriocin producing LAB strains were characterized using phenotypic based clustering method. Data were examined using similarity coefficient (S_J), and unweighted pair group algorithm with arithmetic averages (UPGMA). Seven clusters with other two members were defined at the 79 % S_{SM}-similarity level. The clusters were designated *Lactobacillus plantarum* (cluster A), *Lactobacillus plantarum*, *Lactobacillus* sp. (cluster C), *Pediococcus pentsaceus* (cluster E) and heterofermentative *Lactobacillus* sp. (cluster F). The following species were characterized; *Lb. plantarum*, *Lb. fermentum*, *Lc. lactis*, *Pediococcus acidilactici*, *Pe. pentosaseus*, and unidentified lactobacilli. Partial sequencing of the 16S rRNA gene of representative isolates from each cluster was performed, and ten strains were assigned to seven species: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus ingluviei*, *Pediococcus pentosaceus*, *Lactobacillus acidipiscis* and *Weissella cibaria*. The molecular method used failed to determine the exact taxonomic status of two strains (BH0900 and AH3133). Further taxonomic studies using other molecular based methods are recommended.

In addition, selection of potentially probiotic *Lactobacillus* strains (mainly based on the resistance to the acidity and bile salts) leads to the fact that *Lactobacillus plantarum*, and *Lb. fermentum* strains were more resistant to the action of these harsh conditions. At pH 2, only tow strains (*Lactobacillus* sp. BH1398 and BH1480) that could not recovered after three hours. *Lb. plantarum*, *Lb. fermentum*, *Lb. acidophyllus*, and *Lb. pentosus* proved to be the most resistant strains, with losses in cell viability ranging from 0.77 to 4.04 log orders. In addition, at pH 3 all strains could grow and resist the acidic conditions. The log cfu/ml ranged from 2.73 ± 0.33 to 9.30 ± 0.31, with losses in cell viability ranging from 0.40 to 3.61 log orders for most strains. It seems that, 0.3 % and 0.5 % of bile salts does not affect greatly the survival of most strains. Survival ranged from 81.0 ± 3.5 to 93.5 ± 3.9 %. In contrast, in the presence of 1.0 % bile salts, survival of five strains was decreased by more than 50 %. *Lactobacillus fermentum* BH1509 was considered the most tolerant strain (77.5 % for 1 % bile) followed by *Lactobacillus plantarum* BH1541 (59.9 % for 1 % bile). Furthermore, in the end of this thesis, *Lactobacillus fermentum* BH1509 strain inhibits the growth of Helicobacter pylori strains *in vitro*. The inhibition is resulted by the production of a low molecular weight bacteriocin designated Bac-F15. The bacteriocin has a wide spectrum of action under a wide pH range. Further studies on the interaction of *Lactobacillus fermentum* BH1509 or its bacteriocin with *Helicobacter pylori in vitro* and *in vivo* should be addressed.

FUTURE RESEARCH DIRECTIONS

In Algeria, lactic acid bacteria are mainly associated with fermented dairy products such as cheese, buttermilk and yoghurt. They are also associated with beneficial health effects. Actually, an increasing number of health food as so-called functional food as well as pharmaceutical preparations are promoted with health claims based on the characteristics of certain strains of lactic acid bacteria. There has been increasing range of probiotic products produced of dairy origin such as yoghurt, fermented milks and other dairy products while consumption of tablets or capsule containing freeze-dried probiotic organisms have become increasingly popular due to their higher bacterial concentrations.

While knowledge of some probiotic strains for their acid, bile tolerance and bile deconjugation ability has been ascertained as well as antimicrobial ability and anti-carcinogenic properties, further work is still needed on developing of new and efficient probiotic strains and determining their suitable concentration for daily consumption in order to achieve health benefits.

The inhibitory ability of probiotic bacteria especially lactobacilli towards *Helicobacter pylori* has important clinical implications. Due to the exponential increase in resistance to antibiotics by many bacterial species, research on probiotics is imperative to successfully demonstrate effectiveness in reduction and elimination of these strains from human stomach.

Our work has shown that several *Lactobacillus* fermentum strain BH1509 is able to successfully inhibit *Helicobacter pylori* in vitro. These strain need to be further assessed and critically evaluated by clinical research on patients colonized and infected with *Helicobacter pylori*. It may be useful to study the combination of this strain with antibiotics for the most successful in eliminating *H. pylori in vivo*. Or without the use of antibiotics, which cause disturbance in the balance of the gastrointestinal microflora.

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This is a refereed journal and all articles are professionally screened and reviewed **ORIGINAL ARTICLE**

Microbiological Sanitary Aspects of Pollen

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ABSTRACT

Medicinal plants have been used for centuries as remedies against human diseases because they contain components of therapeutic value. Bee-Pollen was used for thousands of years as functional food and medicinal plant product. Various beneficial effects were attributed to it and its consumption was increased years. The positive effects of a functional food can be either maintaining a state of wellbeing and health or reducing the risk of pathologic consequences. Gastrointestinal functions, redox and antioxidant systems, and metabolism of macronutrients are the most promising targets for functional food science. Negative and undesirable side effects of food are important threads for human health, especially, unprocessed functional foods. In this work, sanitary microbiological analysis of unprocessed pollen collected from different Algerian regions was examined. Results obtained indicate that pollen obtained from Blida is more contaminated than samples obtained from Sétif and Biskra. Total aerobic count was 3,0 ; 5,80 ; and 5,67 Log_{10} cfu/g for Blida, sétif and Biskra respectively. However, the occurrence of *Staphylococcus aureus* was observed only with pollen obtained from Sétif region. Total coliforms count increases respectively with the increase of environmental humidity and temperature (3,0; 4,27 ; and 5,47 Log_{10} cfu/g for Biskra, sétif and Blida respectively). This phenomenon was also noticed for total moulds. From this primary microbiological analysis, consumption of unprocessed pollen can present a challenge for human and animal health, as it could be a carrier for numerous microorganisms, which may present a detrimental effects on body health.

***Key words*:** bee-pollen, medicinal plants, functional foods, microorganisms, microbial quality, mycotoxins, endotoxin.

Introduction

Stamens are the male reproductive organs of flowering plants. They consist of an anther, the site of pollen development, and in most species a stalk-like filament, which transmits water and nutrients to the anther and positions it to aid pollen dispersal. Within the anther, male sporogenous cells differentiate and undergo meiosis to produce microspores, which give rise to pollen grains, whereas other cell types contribute to pollen maturation, protection, or dispersion [1]. Faegri and Van der Pijl [2], defined two major types of pollen dispersal: *Biotic pollination* in which the pollen dispersal agent is an animal (i.e., either an invertebrate or a vertebrate); and *abiotic pollination* where pollen is dispersed by an inanimate physical agent, such as wind or water. Mutually beneficial ecological relationships have been established between bees and plants. There are about 250 thousand species of flowering plants on earth, many of which have amazingly complex relationships to bees and other pollinators including flies, beetles, moths, butterflies, birds and bats [3]. Honeybees, mainly *Apis mellifera*, remain the most economically valuable pollinators of crop monocultures worldwide and yields of some fruit, seed and nut crops decrease by more than 90% without these pollinators [4]. Honeybees remove pollen from an anther by using their tongue and mandibles mixed it with salivary secretions from her mouth, and transfer it to the corbicula, or "pollen basket", on her posterior pair of legs. To prevent bacterial growth and delay pollen germination various enzymes produced by worker bees are added to the pollen as it is packed into broad-free combs [5]. Historically, bee-collected pollen has been used as a food and medicine by various civilizations, as different nutritional and medicinal traits were attributed to it [6]. Nowadays, tons of pollen, either processed or unprocessed, are

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sold for human and animal consumption. Pollen is a riche plant product, containing carbohydrates, proteins, enzymes, fatty acids, minerals, and vitamins. However, unlike honey, from the anther dehiscence to comb cells, pollen is exposed for microbial contamination. This contamination can be attributed to various factors and sources, honey bees, weather, plant materials, insects and animals, humans and their agricultural devices. Numerous studies were revealed the occurrence of fungal and bacterial species in pollen, but little is known about its microbiological quality. For this reason, pollen quality control is of utmost importance, particularly if the pollen is intended for human consumption. The aim of this preliminary investigation is to examine microbial quality of tow pollens collected by honeybees and one hand collected pollen from Algeria.

Materials And Methods

Sampling and storage:

Pollen samples were collected from different regions in Algeria during flowering season (from March to May 2010). Pollen sample 1 (PS1) was collected from Sétif (North east of Algeria), pollen sample 2 (PS2) was from Biskra (North Algerian Sahara), whereas pollen sample 3 (PS3) was from Blida (North of Algeria). Samples were obtained from clean ecological regions situated in agricultural lands. PS1 and PS 3 were trapped from honey bees workers, whereas, PS2 was collected from date palm trees by hands. All the samples were transported to the laboratory in sterile glass vials and conserved at 4°C until use.

Microbial and weather parameters analysis:

Twenty-five grams of each sample of pollen were diluted in 225 mL of peptone saline solution (1 g/L peptone and 8.5 g/L NaCl) and homogenized for 10 min by shaking in an orbitrary shaker (200 rpm). After 30 min at room temperature, serial dilutions of suspension were made in a saline solution (8.5 g/L NaCl) and analyzed. The formed colonies on the plates were counted and expressed as log colony forming units/g (log cfu/g). Another 25 g were diluted in 225 mL of buffered peptone water for the isolation of *Salmonella spp*.

Total Aerobic microflora was determined by spreading 0.1 ml from the appropriate dilution onto sterile disposable Petri dishes to which Plate count Agar (Merck, 5463) was poured. Plates were incubated aerobically at 30° C and counted after 3 days.

Total Anaerobic microflora: The plat count agar was used to enumerate anaerobic microflora by spreading 100 µl from the appropriate dilution on plate count agar. The plates were incubated at 30°C for 3 days under anaerobic conditions using candle jars.

Total Mold and Yeasts From appropriate dilutions. 100 µl were surface plating onto sterile disposable Petri dishes to which was added Potatoes Dextrose agar (SIGMA, P2181). Plates were incubated at 28°C and counted after 7 days.

Total Coliforms: A 100 µl aliquot of the appropriate dilution were transferred into Petri plates and poured with Violet Red Bile Glucose agar (VRBG Agar). Solidified plates were overlaid with 5 ml of VRBG agar. Plates were incubated at 35°C and typical colonies (dark red, 0.5 mm. or more in diameter) were counted after 24 hr.

Staphylococcus aureus was determined by spreading of 100 µl from the appropriate dilutions on Baird-Parker agar (Merck, 5406) and incubated at 37 °C for 48 h.

Salmonella spp.: Salmonella spp. were determined Pre-enrichment was conducted from samples diluted in 225 ml buffered peptone water incubated at 37°C for 18 hours. Secondary selective enrichment was performed in Rappaport-Vassiliadis peptone broth (41°C for 24 h) and Muller-Kaufmann tetrathionate broth (37°C for 24 h), and plating on Brilliant Green Agar and XLD agar incubated at 37°C for 24 h.

Weather parameters (temperature, relative humidity and precipitation amounts) as well as topographic indicators (Latitude, Longitude, and Altitude) data were obtained from three weather stations [Blida, 603900 (DAAG); Biskra, 605250, (DAUB); and Sétif, 604450 (DAAS)] (Table 1 and Figure 1).

Table1 : Topographic data of the nearest weather stations for sampling sites

Sampling site	Weather station	Latitude	Longitude	Altitude
Blida	603900 (DAAG)	36.68	3.25	25 m
Biskra	605250 (DAUB)	34.8	5.73	87 m
Sétif	604450 (DAAS)	36.18	5.41	1038 m

Results:

Microbial content of three pollen samples is presented in Table 2. It should be emphasized that microbial enumeration varies considerably. Statistical analysis of the data showed that total aerobic counts were significantly higher in pollens from Sétif and Blida (620 x 10^3 and 470 x 10^3 cfu/g respectively), significantly lower in pollen from Biskra (10^3 cfu/g). Similarly, total mould counts were 400 x 10^3/g for pollen from Sétif, 50 x 10^3/g for pollen from Biskra and more than 200 x 10^3/g for pollen from Blida. *Salmonella* was not detected in all samples, whereas, staphylococci were recoded only

in pollen from Blida (27.3 x 10^3/g). Like total aerobic counts and mould counts, higher levels of coliforms were observed in pollen from Sétif (300 x 10^3/g), 19x 10^3/g and 10^3/g for Blida and Biskra respectively. Statistical analysis shows that enumeration of the different microbial groups within each sample has extremely significant differences (P< 0.0001), either for normal set or transformed data. Also, from region to region or from sample to another, microbial content differs greatly (P = 0, 0037; < 0.05). Considering now the weather conditions (Fig. 1), Significant differences in relative humidity and precipitation amount (P <0.05) were recorded between the regions Sétif/Biskra and Blida/Biskra, whereas, there was no statistical differences between Sétif and Blida. In addition, temperature does not differs between all area sampling (P >0.05). Analysis of correlation between the organisms enumerated showed that total aerobic plat counts correlated (r = 0.34) only with total coliforms and relative humidity (RH) (r = 0.12) in pollen from Sétif and correlated (r = 0.31) with total coliforms and (r= 0.69 and r= 0.69) with RH and precipitation amount respectively in pollen from Biskra. In Blida's pollen total aerobic plat counts correlated (r = 025) with staphylococci and both (r = 0.74 and r = 0.47) with relative humidity and precipitation amount. In addition, total molds and yeast counts correlated (r = 0.58) with temperature in pollen from Biskra, and in pollen from Blida correlated (r = 0.37) with relative humidity.

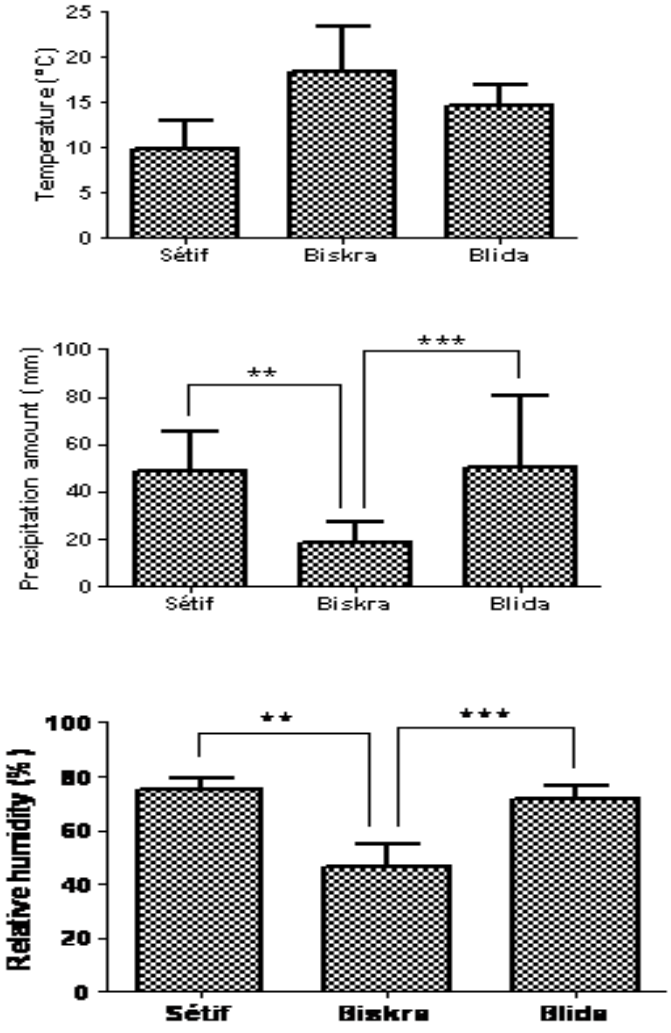

Fig. 1: Recorded temperature (°C), precipitation amount (mm) and relative humidity (%), at the sampling sites (sétif, Biskra, and Blida), columns represent means of five values, upper bares indicate SD (standard deviation), (***) and (**) indicates that P was <0.01 between represented data.

Table 2: Microbial enumeration of three pollen samples collected in Algeria from March to May 2010. (Results were expressed as cfu/g or Log_{10} cfu/g of wet pollen).

	Origin of Pollen Samples					
	PSa (Sétif)		PSb (Biskra)		PSc (Blida)	
	10^3 cfu/g	Log_{10} cfu/g	10^3 cfu/g	Log_{10} cfu/g	10^3 cfu/g	Log_{10} cfu/g
Total aerobic counts	620	5,80	01	3,00	470	5,67
Total anaerobic counts	600	5,77	00	0,00	82	4,91
Moulds and Yeasts	400	5,60	50	4,69	230	5,36
Staphylococcus aureus	00	0,00	00	0,00	27.3	4,43
Salmenella	00	0,00	00	0,00	00	0,00
Total coliforms	300	5,47	01	3,00	19	4,27

Discussion:

Microbial contamination of herbs and/or products may results from improper handling during production, collection and packaging [7]. World Health Organization [8] contaminant guidelines propose that contamination should be avoided and controlled through quality assurance measures such as good agricultural and collection practices (GACP) for medicinal plants, and good manufacturing practices (GMP) for herbal medicines. In recent years, only a small percentage of medicinal plants are collected from the wild, and there are too few data to compare biological contamination between wild and cultivated medicinal herbs. Guidelines such as the GACP and GMP aim at reducing the overall risk of contamination, not only biological [7]. According to Campos et al. [9], pollens should have the following microbial aspects: absence of *Salmonella*/10g; absence of *Staphylococcus* and *Escherichia coli* /01 g; TAPC could not exceed than 10^5 cfu /g; TMYC should be less than 5. 10^4 cfu/g; and the maximum of enterobacteria is 100 cfu/g. Comparing, however, these recommendations with our results reveals clearly that bee-collected pollens (Sétif and Blida) have a poor microbial quality, in contrast to hand collected pollen (Biskra) which have an acceptable criteria. In Algeria, peoples consume pollens. Great populations purchase it from beekeepers and use it as dietary supplement, mainly with dairy products such as milk. However, numerous studies have been conducted to determine microorganisms associated with pollen. Gilliam et al. [10] identified more than six yeast genera (*Cryptococcus, Kloeckera, Candida, Rhodotorula, Torulopsis, Hansenela*) from almond pollen. Later [11] *Bacillus subtilis, B. megaterium, B. licheniformis, and B. circulans* were isolated from the same pollen of almonds. In addition, the majority of molds identified from pollen by Gilliam et al. [12] were penicillia, mucorals and aspergilli. Inside anther pollen is sterile, thus its microbial contamination can be attributed to plant materials, environment, insects (eg, Honeybees), and humans and their agricultural devices. Naturally, pollen grains contain antimicrobial substances (flavonoids, phenolic acids, and other phytochemicals) [13,14] as well as microbial spore germination inhibitors [15]. As reviewed by Gonzâlez et al. [16], growth and sporulation of fungi, both on standing crops and in stored grains, are largely dependent on environmental factors. The most important determinants are probably a_w and temperature. On this fact, results of this work point out that differences of microbial content between and within pollen samples were related to relative humidity and precipitation amount, as temperature does not differs greatly between regions. World wide, pollen processing includes harvesting, drying, cleaning, packaging and storage [17]. Gonzalez et al. [16] concluded in their work that the most critical stage is pollen collection from traps. The longer period of collection the highly contaminated pollen was obtained. In contrast, based on the fact that corbicular pollen is mixture of pollen, nectar and salivary secretions of honeybees [18]. Furthermore, Gilliam et al. [19] reported the occurrence of both Gram negative and Gram positive bacilli as well as Gram positive cocci in nectar. In addition, fungi are found in association with honey bee colonies where they persist on nectar, pollen, in colony debris, and inside bees themselves [20]. And from the results of this work, it seems that honeybees are the main source and/or carrier for pollen contamination. The high incidence of fungi in pollen grains studied in this work point out that raw pollen in not ready-to-eat directly without processing (drying). In Algeria, pollen is dried naturally by exposing it to sunlight or oven heated at a low temperature (less than 45°C). Serra and Alegret [21] recommend the avoidance of natural pollen drying, because at low temperatures, fungal growth and mycotoxin production might occur. If raw pollen was stored without processing, fungi can flourish under appropriate conditions (humidity and temperature) as pollen is a suitable plant product either for fungal growth or mycotoxin production. However, mycotoxins production, such as ochratoxin A (OTA) and aflatoxins B_1, B_2, G_1, and G_2 by pollen associated fungi is known [16, 22]. Aflatoxins and ochratoxin A are thermostable and carcinogenic molecules [23, 22], thus, pollen dried naturally or not may present a threat for human and animal health. Considering now bacterial content, however, the incidence of staphylococci in pollen from Blida make it undesirable dietary supplement. *Staphylococcus aureus* remains a versatile and dangerous pathogen in humans. The microoorganism produce numerous toxins that are responsible for diverse syndromes and life-threatening diseases, as the staphylococci are well known for their multiresistance to antimicrobial drugs [24, 25]. It is

truly that analyzed pollen samples in this work contain high levels of enterobacteria and exceed recommended values. *Enterobacteriaceae* are a group of bacteria that can be found in many environments. They can be found in the intestinal tract of humans and animals. They can also be found in soil, vegetable matter and marine environments. The group includes both pathogenic and non-pathogenic bacteria. As they can be found in raw foods, their detection may not be an indication of fecal contamination and is inappropriate to test ready-to-eat foods containing raw components (particularly fresh fruits and vegetables) [26, 27, 28]. But taking in mind the fact that enterobacterial cell membrane contains an antigenic, pyrogenic and thermostable molecule, the endotoxin or lipopolysaccharide (LPS). However, the bacterial endotoxin is released during bacterial multiplication or death and triggers a series of important biological events that lead to an inflammatory response and bone resorption [26]. Indeed, either raw or dried pollen may be considered as a potentially hazardous medicinal plant product, as it is exposed for contamination by toxigenic fungi and pathogenic bacteria such as aspergilli, penicillia and staphylococci. This contaminated pollen present a suitable environment and/or a suitable delivery system for mycotoxins and/or bacterial endotoxins. Peoples, especially in developing countries, continue to use pollen without the existence of international microbiological quality guidelines and reglementations for pollen, as well as the ineffectiveness of pollen sanitization methods designed for human consumption, more studies are needed to understand the microbial content of pollen and to establish international microbiological quality parameters and standard processing protocols.

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Chapter 8

Lactic Acid Bacteria as Probiotics: Characteristics, Selection Criteria and Role in Immunomodulation of Human GI Muccosal Barrier

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Additional information is available at the end of the chapter

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1. Introduction

As it was reported by Chow (2002), the notion that food could serve as medicine was first conceived thousands of years ago by the Greek philosopher and father of medicine, Hippocrates, who once wrote: 'Let food be thy medicine, and let medicine be thy food'. However, during recent times, the concept of food having medicinal value has been reborn as 'functional foods'. The list of health benefits accredited to functional food continues to increase, and the gut is an obvious target for the development of functional foods, because it acts as an interface between the diet and all other body functions. One of the most promising areas for the development of functional food components lies in the use of probiotics and prebiotics which scientific researches have demonstrated therapeutic evidence. Nowadays, consumers are aware of the link among lifestyle, diet and good health, which explains the emerging demand for products that are able to enhance health beyond providing basic nutrition. Besides the nutritional valaes, ingestion of lactic acid bacteria (LAB) and their fermented foods has been suggested to confer a range of health benefits including immune system modulation, increased resistance to malignancy, and infectious illness (Soccol, et al., 2010). LAB were first isolated from milk. They can be found in fermented products as meat, milk products, vegetables, beverages and bakery products. LAB occur naturally in soil, water, manure, sewage, silage and plants. They are part of the microbiota on mucous membranes, such as the intestines, mouth, skin, urinary and genital organs of both humans and animals, and may have a beneficial influence on these ecosystems. LAB that grow as the adventitious microflora of foods or that are added to foods as cultures are generally considered to be harmless or even an advantage for human

health. Since their discovery, LAB has been gained mush interest in various applications, as starter cultures in food and feed fermentations, pharmaceuticals, probiotics and as biological control agents. In food industry, LAB are widely used as starters to achieve favorable changes in texture, aroma, flavor and acidity (Leory and De Vuyst, 2004). However, there has been an important interest in using bacteriocin and/or other inhibitory substance producing LAB for non-fermentative biopreservation applications. Du to their antimicrobial and antioxidant activities some LAB strains are used in food biopreservation. However, LAB are generally regarded as safe (GRAS) to the consumer and during storage, they naturally dominate the microflora of many foods (Osmanağaoğlu and Beyatli, 1999; Parada et al., 2007). Many of the indications for probiotic activity have been obtained from effects observed in various clinical situations. Even, there are few strains that have officially gained the status of pharmaceutical preparation; each of these effects is gradually being supported by a number of clinical studies or human intervention trials, performed in a way that resembles the traditional pharmacological approach (placebo-controlled, double blind, randomized trials) and the strains used in these studies belong to different microbial species, but are mostly lactic acid bacteria (Mercenier et al, 2003).

2. LAB as probiotic agents

2.1. Overview of probiotics

The most tried and tested manner in which the gut microbiota composition may be influenced is through the use of live microbial dietary additions, as probiotics. In fact, the concept dates back as far as prebiblical ages. The first records of ingestion of live bacteria by humans are over 2,000 years old. However, at the beginning of this century probiotics were first put onto a scientific basis by the work of Metchnikoff (1908). He hypothesised that the normal gut microflora could exert adverse effects on the host and that consumption of 'soured milks' reversed this effect. The word ''probiotics'' was initially used as an anonym of the word ''antibiotic''. It is derived from Greek words pro and biotos and translated as ''for life''. The origin of the first use can be traced back to Kollath (1953), who used it to describe the restoration of the health of malnourished patients by different organic and inorganic supplements. Later, Vergin (1954) proposed that the microbial imbalance in the body caused by antibiotic treatment could have been restored by a probiotic rich diet; a suggestion cited by many as the first reference to probiotics as they are defined nowadays. Similarly, Kolb recognized detrimental effects of antibiotic therapy and proposed the prevention by probiotics (Vasiljevic and Shah, 2008) Later on, Lilly and Stillwell (1965) defined probiotics as "…microorganisms promoting the growth of other microorganisms". Following recommendations of a FAO/WHO (2002) working group on the evaluation of probiotics in food, probiotics, are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Sanders, 2008; Schrezenmeir and De Vrese, **2001)**. The idea of health-promoting effects of LAB is by no means new, as Metchnikoff proposed that lactobacilli may fight against intestinal putrefaction and contribute to long life. Such microorganisms may not necessarily be constant inhabitants of the gut, but they should have a "…beneficial effect on the general and health status of man and animal"

Probiotic	Human disease in which benefit is shown	Animal model in which benefit is shown
Yeast		
Saccharomyces boulardii	*Clostridium difficile* infection	*Citrobacter rodentium*-induced colitis
Gram-negative bacteria		
Escherichia coli Nissle 1917	NA	DSS-induced colitis
Gram-positive bacteria		
Bifidobacteria bifidum	NA	Rat model of necrotizing enterocolitis
Bifidobacteria infantis	IBS29	NA
Lactobacillus rhamnosus GG (used with lactoferrin)	Sepsis in very low birth weight infants	NA
Lactococcus lactis (engineered to produce IL-10 or trefoil factors)	Crohn's disease	DSS-induced colitis and IL-$10^{-/-}$ mice (spontaneous IBD)
Lactobacillus plantarum 299v	Antibiotic-associated diarrhea	IL-$10^{-/-}$ mice (spontaneous IBD)
Lactobacillus acidophilus	NA	Visceral hyperalgesia 40 and *C. rodentium*-induced colitis
Lactobacillus rhamnosus	Pediatric antibiotic-associated diarrhea	–
Lactobacillus casei	NA	DNBS-induced colitis
Bacillus polyfermenticus	NA	DSS-induced colitis and TNBS-induced colitis
Combination regimens		
Lactobacillus rhamnosus GG combined with *Bifidobacterium lactis*	Bacterial infections	NA
Lactobacillus rhamnosus combined with *Lactobacillus helveticus*	NA	*C. rodentium*-induced colitis, chronic stress, and early life stress
VSL#3 (*Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium longum, Bifidobacterium breve, Bifidocacterium infantis and Streptococcus thermophilus*)	Pouchitis and pediatric ulcerative colitis	DSS-induced colitis, IL-$10^{-/-}$ mice (spontaneous IBD; DNA only), and SAMP mouse model of spontaneous IBD

Abbreviations: DNBS, dinitrobenzene sulfonic acid; DSS, dextran sodium sulfate; IL-10, interleukin 10; NA, not available; TNBS, trinitrobenzene sulfonic acid.

Table 1. Selected organisms that are used as probiotic agents (Gareau et al., 2010).

(Holzapfel et al., 2001; Belhadj et al., 2010). Other definitions advanced through the years have been restrictive by specification of mechanisms, site of action, delivery format, method, or host. Probiotics have been shown to exert a wide range of effects. The mechanism of action of probiotics (e.g, having an impact on the intestinal microbiota or enhancing immune function) was dropped from the definition to encompass health effects due to novel mechanisms and to allow application of the term before the mechanism is confirmed. Physiologic benefits have been attributed to dead microorganisms. Furthermore, certain mechanisms of action (such as delivery of certain enzymes to the intestine) may not require live cells. However, regardless of functionality, dead microbes are not probiotics (Sanders, 2008). In relation to food, probiotics are considered as "viable preparations in foods or dietary supplements to improve the health of humans and animals". According to these definitions, an impressive number of microbial species are considered as probiotics. (Holzapfel et al., 2001). For gastrointestinal ecosysteme, however, the most important microbial species that are used as probiotics are lactic acid bacteria (LAB) (Table 1).

2.2. Selection of probiotics

Many in vitro tests are performed when screening for potential probiotic strains. The first step in the selection of a probiotic LAB strain is the determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain. All these characteristics have important consequences on the selection of the novel strains (Morelli, 2007). An FAO/WHO (2002) expert panel suggested that the specificity of probiotic action is more important than the source of microorganism. This conclusion was brought forward due to uncertainty of the origin of the human intestinal microflora since the infants are borne with virtually sterile intestine. However, the panel also underlined a need for improvement of in vitro tests to predict the performance of probiotics in humans. While many probiotics meet criteria such as acid and bile resistance and survival during gastrointestinal transit, an ideal probiotic strain remains to be identified for any given indication. Furthermore, it seems unlikely that a single probiotic will be equally suited to all indications; selection of strains for disease-specific indications will be required (Shanahan, 2003).

The initial screening and selection of probiotics includes testing of the following important criteria: phenotype and genotype stability, including plasmid stability; carbohydrate and protein utilization patterns; acid and bile tolerance and survival and growth; intestinal epithelial adhesion properties; production of antimicrobial substances; antibiotic resistance patterns; ability to inhibit known pathogens, spoilage organisms, or both; and immunogenicity. The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization (Tuomola et al., 2001). The table below (Table 2) indicates key creteria for sellecting probiotic candidat for commercial application, and figure 1 presents major and cardinal steps for sellecting probiotic candidats.

It is of high importance that the probiotic strain can survive the location where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the strain can

proliferate and colonise at this specific location. Probably only host-specific microbial strains are able to compete with the indigenous microflora and to colonise the niches. Besides, the probiotic strain must be tolerated by the immune system and not provoke the formation of antibodies against the probiotic strain. So, the host must be immuno-tolerant to the probiotic. On the other hand, the probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms. It goes without saying that a probiotic has to be harmless to the host: there must be no local or general pathogenic, allergic or mutagenic/carcinogenic reactions provoked by the microorganism itself, its fermentation products or its cell components after decrease of the bacteria (Desai, 2008).

General	**Property**
Safety criteria	Origin
	Pathogenicity and infectivity
	Virulence factors—toxicity, metabolic activity and intrinsic properties, i.e., antibiotic resistance
Technological criteria	Genetically stable strains
	Desired viability during processing and storage
	Good sensory properties
	Phage resistance
	Large-scale production
Functional criteria	Tolerance to gastric acid and juices
	Bile tolerance
	Adhesion to mucosal surface
	Validated and documented health effects
Desirable physiological criteria	Immunomodulation
	Antagonistic activity towards gastrointestinal pathogens, i.e., Helicobacter pylori, Candida albicans
	Cholesterol metabolism
	Lactose metabolism
	Antimutagenic and anticarcinogenic properties

Table 2. Key and desirable criteria for the selection of probiotics in commercial applications (Vasiljevic and Shah, 2008).

When probiotic strains are selected, attributes important for efficacy and technological function must be assessed and a list of characteristics required for all probiotic functions is required. Basic initial characterization of strain identity and taxonomy should be conducted, followed by evaluation with validated assays both in studies of animal models and in controlled studies in the target host. In vitro assays are frequently conducted that have not been proved to be predictive of in vivo function. Technological robustness must also be determined, such as the strain's ability to be grown to high numbers, concentrated, stabilized, and incorporated into a final product with good sensory properties, if applicable, and to be stable, both physiologically and genetically, through the end of the shelf life of the product and at the active site in the host. Assessment of stability can also be a challenge, since factors such as chain length and injury may challenge the typical assessment of colony-

forming units, as well as in vivo function (Sanders, 2008). Dose levels of probiotics should be based on levels found to be efficacious in human studies. One dose level cannot be assumed to be effective for all strains. Furthermore, the impact of product format on

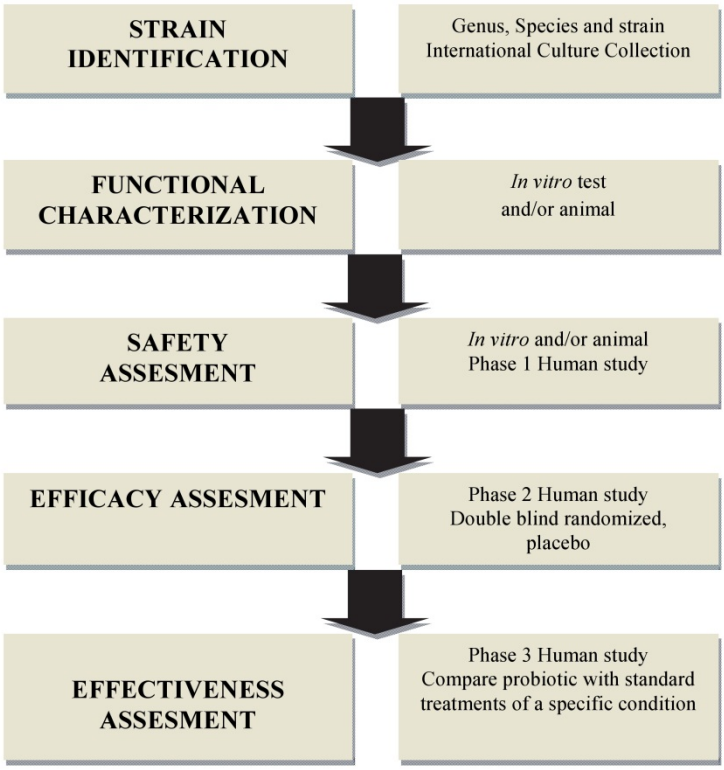


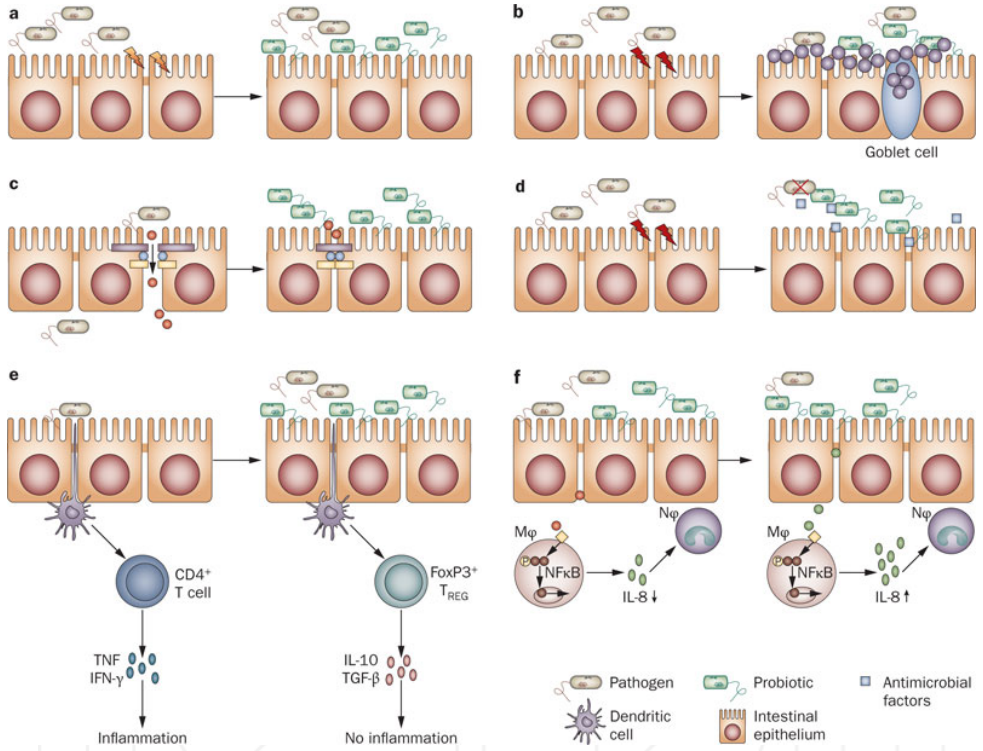
Figure 1. Scheme of the Guidelines for the Evaluation of Probiotics for Food Use. (Adapted from, Collado et al., 2009).

probiotic function has yet to be explored in depth. The common quality-control parameter of colony-forming units per gram may not be the only parameter indicative of the efficacy of the final product. Other factors, such as probiotic growth during product manufacture, coating, preservation technology, metabolic state of the probiotic, and the presence of other functional ingredients in the final product, may play a role in the effectiveness of a product. More research is needed to understand how much influence such factors have on in vivo efficacy **(Sanders, 2008).**

2.3. Potential mechanisms of action of probiotics

A wide variety of potential beneficial health effects have been attributed to probiotics (Table 3). Claimed effects range from the alleviation of constipation to the prevention of major life-threatening diseases such as inflammatory bowel disease, cancer, and cardiovascular

incidents. Some of these claims, such as the effects of probiotics on the shortening of intestinal transit time or the relief from lactose maldigestion, are considered well-established, while others, such as cancer prevention or the effect on blood cholesterol levels, need further scientific backup (Leroy et al., 2008). The mechanisms of action may vary from one probiotic strain to another and are, in most cases, probably a combination of activities, thus making the investigation of the responsible mechanisms a very difficult and complex task. In general, three levels of action can be distinguished: probiotics can influence human



Probiotic organisms can provide a beneficial effect on intestinal epithelial cells in numerous ways. **a**: Some strains can block pathogen entry into the epithelial cell by providing a physical barrier, referred to as colonization resistance or **b**: create a mucus barrier by causing the release of mucus from goblet cells. **c**: Other probiotics maintain intestinal permeability by increasing the intercellular integrity of apical tight junctions, for example, by upregulating the expression of zona-occludens 1 (a tight junction protein), or by preventing tight junction protein redistribution thereby stopping the passage of molecules into the lamina propria. **d**: Some probiotic strains have been shown to produce antimicrobial factors. **e**: Still other strains stimulate the innate immune system by signaling dendritic cells, which then travel to mesenteric lymph nodes and lead to the induction of TREG cells and the production of anti-inflammatory cytokines, including IL-10 and TGF-β. f: Some probiotics (or their products) may also prevent (left-hand side) or trigger (right-hand side) an innate immune response by initiating TNF production by epithelial cells and inhibiting (or activitating) NFκB in Mϕ and dampening (or priming) the host immune response by influencing the production of IL-8 and subsequent recruitment of Nϕ to sites of intestinal injury. Abbreviations: Mϕ, macrophage; Nϕ, neutrophil; TREG cell, regulatory T cell. Reproduced from, *Gareau M. G., P. M. Sherman & W. A. Walker (2010) Nature Reviews Gastroenterology and Hepatology 7, 503-514.*

Figure 2. Potential mechanisms of action of probiotics.

Health benefit	Proposed mechanism(s)
Cancer prevention	Inhibition of the transformation of pro-carcinogens into active carcinogens, binding/inactivation of mutagenic compounds, production of anti-mutagenic compounds, suppression of growth of pro-carcinogenic bacteria, reduction of the absorption of carcinogens, enhancment of immune function, influence on bile salt concentrations
Control of irritable bowel syndrome	Modulation of gut microbiota, reduction of intestinal gas production
Management and prevention of atopic diseases	Modulation of immune response
Management of inflammatory bowel diseases (Crohn's disease, ulcerative colitis, pouchitis)	Modulation of immune response, modulation of gut microbiota
Prevention of heart diseases/influence on blood cholesterol levels	Assimilation of cholesterol by bacterial cells, deconjugation of bile acids by bacterial acid hydrolases, cholesterol-binding to bacterial cell walls, reduction of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to liver through influence of the bacterial production of short-chain fatty acids
Prevention of urogenital tract disorders	Production of antimicrobial substances, competition for adhesion sites, competitive exclusion of pathogens
Prevention/alleviation of diarrhoea caused by bacteria/viruses	Modulation of gut microbiota, production of antimicrobial substances, competition for adhesion sites, stimulation of mucus secretion, modulation of immune response
Prevention/treatment of *Helicobacter pylori* infections	Production of antimicrobial substances, stimulation of the mucus secretion, competition for adhesion sites, stimulation of specific and non-specific immune responses
Relief of lactose indigestion	Action of bacterial β-galactosidase(s) on lactose
Shortening of colonic transit time	Influence on peristalsis through bacterial metabolite production

Table 3. Potential and established health benefits associated with the usage of probiotics (Leroy et al., 2008).

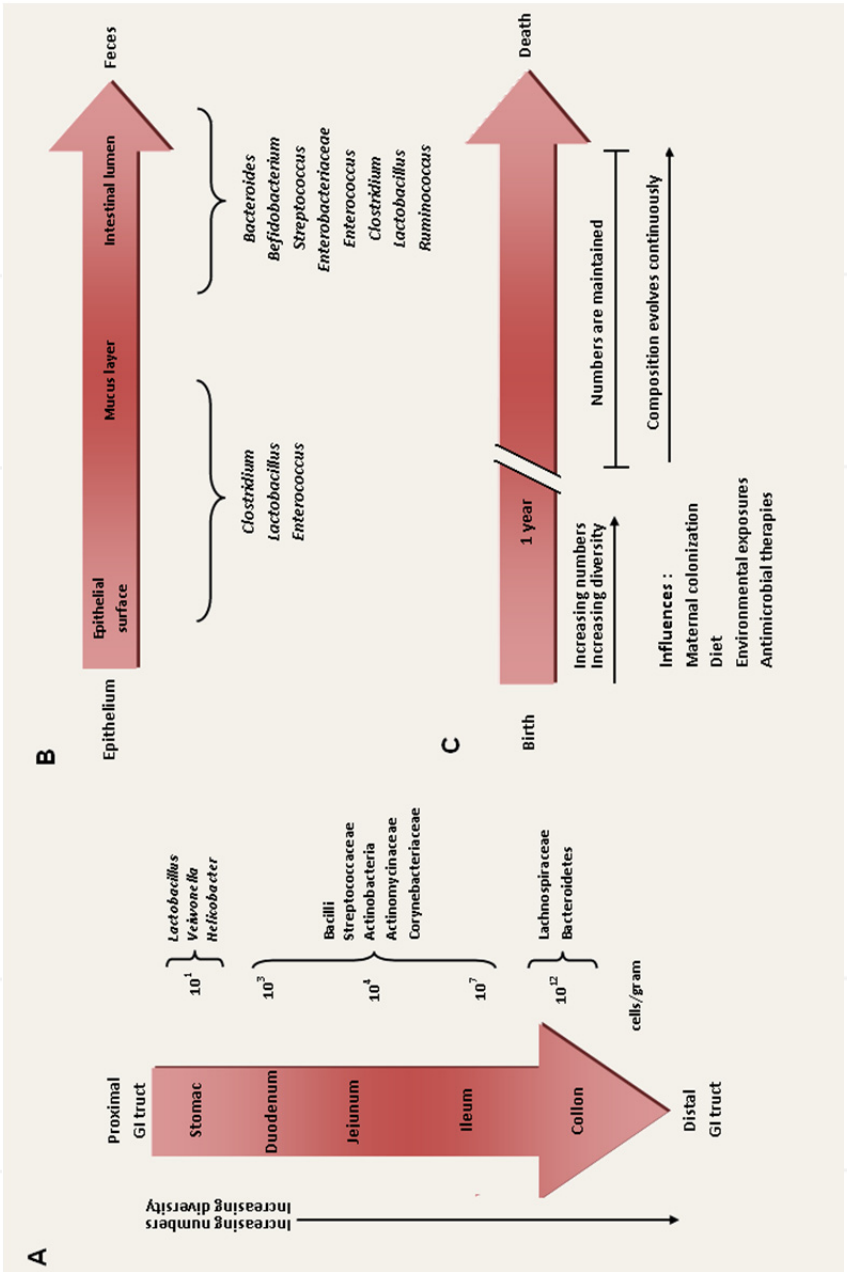
health by interacting with other microorganisms present on the site of action, by strengthening mucosal barriers, and by affecting the immune system of the host (Leroy et al., 2008), and the figure 2 shows the most important mechanisms by whiche probiotics exerce their action inside the gut.

3. Probiotics and gut health

3.1. Gut microbiota

The human gastrointestinal tract is inhabited by a complex and dynamic population of around 500-1000 of different microbial species which remain in a complex equilibrium. It has been estimated that bacteria account for 35–50% of the volume content of the human colon. These include *Bacteroides*, *Lactobacillus*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia and Veillonella*. The bacterial strains with identified beneficial properties include mainly *Bifidobacterium* and *Lactobacillus* species. The dominant microbial composition of the intestine have been shown to be stable over time during adulthood, and the microbial patterns are unique for each individual. However, there are numerous external factors that have potential to influence the microbial composition in the gut as host genetics, birth delivery mode, diet, age, antibiotic treatments and also, other microorganisms as probiotics. (Collado et al., 2009). The intestine is one of the main surfaces of contact with exogenous agents (viruses, bacteria, allergens) in the human body. It has a primary role in the host defense against external aggressions by means of the intestinal mucosa, the local immune system, and the interactions with the intestinal microbiota (resident and in transitbacteria). Gut microbiota influences human health through an impact on the gut defense barrier, immune function, nutrient utilization and potentially by direct signaling with the gastrointestinal epithelium (Collado et al., 2009). Only a limited fraction of bacterial phyla compose the major intestinal microbiota. In healthy adults, 80% of phylotypes belong to four major phylogenetic groups, which are the *Clostiridium leptum*, *Clostridium coccoides*, *Bacteroides* and *Bifidobacteria* groups. However, a large fraction of dominant phylotypes is subject specific. Also, studies have found that mucosal microbiota is stable along the distal gastrointestinal tract from ileum to rectum, but mucosa-associated microbiota is different from fecal microbiota. The difference has been estimated to be between 50–90%.

The intestinal microbiota is not homogeneous. The number of bacterial cells present in the mammalian gut shows a continuum that goes from 10^1 to 10^3 bacteria per gram of contents in the stomach and duodenum, progressing to 10^4 to 10^7 bacteria per gram in the jejunum and ileum and culminating in 10^{11} to 10^{12} cells per gram in the colon (Figure 3a). Additionally, the microbial composition varies between these sites. In addition to the longitudinal heterogeneity displayed by the intestinal microbiota, there is also a great deal of latitudinal variation in the microbiota composition (Figure 3b). The intestinal epithelium is separated from the lumen by a thick and physicochemically complex mucus layer. The microbiota present in the intestinal lumen differs significantly from the microbiota attached and embedded in this mucus layer as well as the microbiota present in the immediate



a: variations in microbial numbers and composition across the length of the gastrointestinal tract. *b*: longitudinal variations in microbial composition in the intestine. *c*: temporal aspects of microbiota establishment and maintenance and factors influencing microbial composition. (Sekirov et al., 2010).

Figure 3. Spatial and temporal aspects of intestinal microbiota composition.

proximity of the epithelium. For instance, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of *Enterobacteriacea*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* were all found in feces, whereas only *Clostridium*, *Lactobacillus*, and *Enterococcus* were detected in the mucus layer and epithelial crypts of the small intestine (Sekirov et al., 2010). Colonization of the human gut with microbes begins immediately at birth (Figure 3c). Upon passage through the birth canal, infants are exposed to a complex microbial population. After the initial establishment of the intestinal microbiota and during the first year of life, the microbial composition of the mammalian intestine is relatively simple and varies widely between different individuals and also with time. However, after one year of age, the intestinal microbiota of children starts to resemble that of a young adult and stabilizes (Figure 3c) (Sekirov et al., 2010).

3.2. Survival and antagonism effects of probiotics in the gut

The intestinal epithelium is the largest mucosal surface in the human body, provides an interface between the external environment and the host. The gut epithelium is constantly exposed to foreign microbes and antigens derived from digested foods. Thus, the gut epithelium acts as a physical barrier against microbial invaders and is equipped with various elements of the innate defense system. In the gut, two key elements govern the interplay between environmental triggers and the host: intestinal permeability and intestinal mucosal defense. Resident bacteria can interact with pathogenic microorganisms and external antigens to protect the gut using various strategies.

According to the generally accepted definition of a probiotic, the probiotic microorganism should be viable at the time of ingestion to confer a health benefit. Although not explicitly stated, this definition implies that a probiotic should survive GI tract passage and, colonize the host epithelium. A variety of traits are believed to be relevant for surviving GI tract passage, the most important of which is tolerance both to the highly acidic conditions present in the stomach and to concentrations of bile salts found in the small intestine. These properties have consequently become important selection criteria for new probiotic functionality. In addition to tolerating the harsh physical-chemical environment of the GI tract, adherence to intestinal mucosal cells would be necessary for colonization and any direct interactions between the probiotic and host cells leading to the competitive exclusion of pathogens and/or modulation of host cell responses. Moreover, As enteropathogenic Escherichia coli are known to bind to epithelial cells via mannose receptors, probiotic strains with similar adherence capabilities could inhibit pathogen attachment and colonization at these binding sites and thereby protect the host against infection (Marco et al., 2006).

Probiotic bacteria can antagonize pathogenic bacteria by reducing luminal pH, inhibiting bacterial adherence and translocation, or producing antibacterial substances and defensins. One of the mechanisms by which the gut flora resists colonization by pathogenic bacteria is by the production of a physiologically restrictive environment, with respect to pH, redox potential, and hydrogen sulfide production. Probiotic bacteria decrease the luminal pH, as has been demonstrated in patients with ulcerative colitis (UC) following ingestion of the

probiotic preparation VSL#3. In a fatal mouse Shiga toxin-producing *E. coli O157:H7* infection model, the probiotic Befidobacterium breve produced a high concentration of acetic acid, consequently lowering the luminal pH. This pH reduction was associated with increased animal survival (Ng et al., 2009).

Production of antimicrobial compounds, termed bacteriocins, by probiotic bacteria is also likely to contribute to their beneficial activity. Several bacteriocins produced by different species from the genus *Lactobacillus* have been described. The inhibitory activity of these bacteriocins varies; some inhibit taxonomically related Gram-positive bacteria, and some are active against a much wider range of Gram-positive and Gram-negative bacteria as well as yeasts and molds. For example, the probiotic *L. salivarius subsp. salivarius* UCC118 produces a peptide that inhibits a broad range of pathogens such as *Bacillus*, *Staphylococcus*, *Enterococcus*, *Listeria,* and *Salmonella* species. Lacticin 3147, a broad-spectrum bacteriocin produced by *Lactococcus lactis*, inhibits a range of genetically distinct *Clostridium difficile* isolates from healthy subjects and patients with IBD. A further example is the antimicrobial effect of *Lactobacillus* species on *Helicobacter pylori* infection of gastric mucosa, achieved by the release of bacteriocins and the ability to decrease adherence of this pathogen to epithelial cells (Gotteland et al., 2006). Probiotics can reduce the epithelial injury that follows exposure to *E. coli O157:H7* and *E. coli O127:H6*. The pretreatment of intestinal (T84) cells with lactic acid-producing bacteria reduced the ability of pathogenic *E. coli* to inject virulence factors into the cells or to breach the intracellular tight junctions. Adhesion and invasion of an intestinal epithelial cell line (Intestine 407) by adherent invasive *E. coli* isolated from patients with Crohn's disease (CD) was substantially diminished by co- or preincubation with the probiotic strain *E. coli* Nissle 1917 (Wehkamp et al., 2004 ; Schlee et al., 2007). These findings demonstrate that probiotics prevent epithelial injury induced by attaching-effacing bacteria and contributes to an improved mucosal barrier and provide a means of limiting access of enteric pathogens (Sherman et al., 2005).

4. Probiotics and the mucous layer

Most mucosal surfaces are covered by a hydrated gel formed by mucins. Mucins are secreted by specialized epithelial cells, such as gastric foveolar mucous cells and intestinal goblet cells, Goblet cells are found along the entire length of the intestinal tract, as well as other mucosal surfaces. Mucins, are abundantly core glycosylated (up to 80% wt/wt) and either localized to the cell membrane or secreted into the lumen to form the mucous layer (Turner, 2009). Of the 18 mucin-type glycoproteins expressed by humans, MUC2 is the predominant glycoprotein found in the small and large bowel mucus. The NH2- and COOH-termini are not glycosylated to the same extent, but are rich in cysteine residues that form intra- and inter-molecular disulfide bonds. These glycan groups confer proteolytic resistance and hydrophilicity to the mucins, whereas the disulfide linkages form a matrix of glycoproteins that is the backbone of the mucous layer (Ohland and MacNaughton, 2010). Although small molecules pass through the heavily glycosylated mucus layer with relative ease, bulk fluid flow is limited and thereby contributes to the development of an unstirred layer of fluid at the epithelial cell surface. As the unstirred layer is protected from

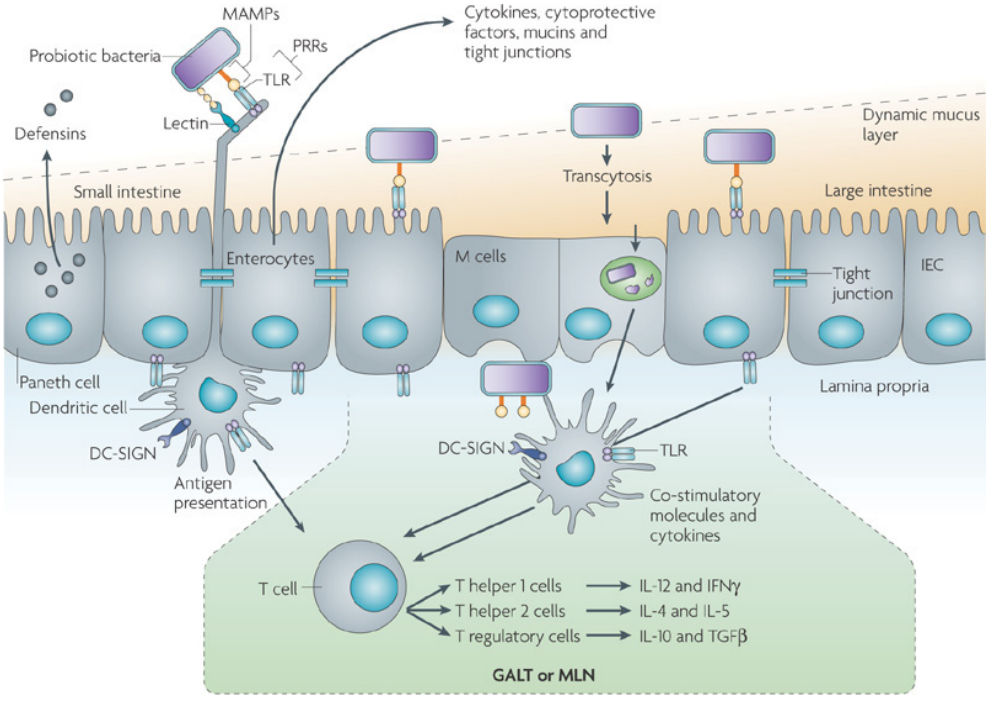
convective mixing forces, the diffusion of ions and small solutes is slowed (Turner, 2009). This gel layer provides protection by shielding the epithelium from potentially harmful antigens and molecules including bacteria from directly contacting the epithelial cell layer, while acting as a lubricant for intestinal motility. Mucins can also bind the epithelial cell surface carbohydrates and form the bottom layer, which is firmly attached to the mucosa, whereas the upper layer is loosely adherent. The mucus is the first barrier that intestinal bacteria meet, and pathogens must penetrate it to reach the epithelial cells during infection (Ohland and MacNaughton, 2010).

Probiotics may promote mucus secretion as one mechanism to improve barrier function and exclusion of pathogens. In support of this concept, probiotics have been shown to increase mucin expression in vitro, contributing to barrier function and exclusion of pathogens. Several studies showed that increased mucin expression in the human intestinal cell lines Caco-2 (MUC2) and HT29 (MUC2 and 3), thus blocking pathogenic *E. coli* invasion and adherence. However, this protective effect was dependent on probiotic adhesion to the cell monolayers, which likely does not occur in vivo (Mack et al., 2003; Mattar et al., 2002). Conversely, another study showed that *L. acidophilus A4* cell extract was sufficient to increase MUC2 expression in HT29 cells, independent of attachment (Kim et al., 2008). Additionally, intestinal trefoil factor 3 (TFF3) is coexpressed with MUC2 by colonic goblet cells and is suggested to promote wound repair (Gaudier et al., 2005 ; Kalabis et al., 2006). However, healthy rats did not display increased colonic TFF3 expression after stimulation by VSL#3 probiotics (Caballero-Franco et al., 2007). Furthermore, mice treated with 1% dextran sodium sulfate (DSS) to induce chronic colitis did not exhibit increased TFF3 expression or wound healing when subsequently treated with VSL#3. This observation indicates that probiotics do not enhance barrier function by up-regulation of TFF3, nor are they effective at healing established inflammation. Therefore, use of current probiotics is likely to be effective only in preventing inflammation as shown by studies in animal models (Ohland and MacNaughton, 2010).

5. Interaction of probiotic bacteria with gut epithelium

The composition of the commensal gut microbiota is probably influenced by the combination of food practices and other factors like the geographical localization, various levels of hygiene or various climates. The host-microbe interaction is of primary importance during neonatal period. The establishment of a normal microbiota provides the most substantial antigenic challenge to the immune system, thus helping the gut associated lymphoid tissus (GALT) maturation. The intestinal microbiota contributes to the anti-inflammatory character of the intestinal immune system. Several immunoregulatory mechanisms, including regulatory cells, cytokines, apoptosis among others, participate in the control of immune responses by preventing the pathological processes associated with excessive reactivity. An interesting premise for probiotic physiological action is their capacity to modulate the immune system. Consequently, many studies have focused on the effects of probiotics on diverse aspects of the immune response. Following consumption of probiotic products, the interaction of these bacteria with intestinal enterocytes initiates a

host response, since intestinal cells produce various immunomodulatory molecules when stimulated by bacteria (Delcenseri et al., 2009). Furthermore, the indigenous microbiota is a natural resistance factor against potential pathogenic microorganisms and provides colonization resistance, also known as gut barrier, by controlling the growth of opportunistic microorganisms. It has been suggested that commensal bacteria protect their host against microbial pathogens by interfering with their adhesion and toxic effects (Myllyluoma, 2007).



A fraction of ingested probiotics are able to interact with intestinal epithelial cells (IECs) and dendritic cells (DCs), depending on the presence of a dynamic mucus layer. Probiotics can occasionally encounter DCs through two routes: DCs residing in the lamina propria sample luminal bacterial antigens by passing their dendrites between IECs into the gut lumen, and DCs can also interact directly with bacteria that have gained access to the dome region of the gut-associated lymphoid tissue (GALT) through specialized epithelial cells, termed microfold or M cells. The interaction of the host cells with microorganism-associated molecular patterns (MAMPs) that are present on the surface macromolecules of probiotic bacteria will induce a certain molecular response. The host pattern recognition receptors (PRRs) that can perceive probiotic signals include Toll-like receptors (TLRs) and the C type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). Some molecular responses of IECs depend on the subtype of cell, for example, Paneth cells produce defensins and goblet cells produce mucus. Important responses of DCs against probiotics include the production of cytokines, major histocompatibility complex molecules for antigen presentation, and co-stimulatory molecules that polarize T cells into T helper or CD4+CD25+ regulatory T cells in the mesenteric lymph nodes (MLNs) or subepithelial dome of the GALT. IFNγ, interferon-γ; IL, interleukin; TGFb; transforming growth factor-β. Reproduced from: *S. Lebeer, J. Vanderleyden & S. C. J. De Keersmaecker (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature Reviews Microbiology 8, 171-184.*

Figure 4. Interaction of probiotic bacteria with IECs and DCs from the GALT.

The tight epithelial cell barrier forms the another line of defence between the gut luminal contents and the host. Epithelial cells lining the gastrointestinal tract are able to respond to infection by initiating either nonspecific or specific host-defence response (Kagnoff and Eckmann 1997, Strober 1998). Bacterial adhesion to the host cell or recognition by the host cell is often an essential first stage in the disease process. A wide range of gastrointestinal cell surface constituents, such as several glygoconjucates, can serve as receptors for bacterial adherence (Servin and Coconnier 2003, Pretzer et al., 2005). Furthermore, epithelial cells express constitutively host pattern recognition receptors (PRRS), such as Toll-like receptors (TLR). These are a family of transmembrane receptors that recognize repetitive patterns, i.e. the pathogen-associated molecular patterns present in diverse microbes, including gram-positive and gram-negative bacteria (Bäckhed and Hornef 2003, Takeda et al., 2003). TLRs are also found on innate immune cells, such as dendritic cells and macrophages (Vinderola et al., 2005). TLR4 recognizes lipopolysaccharide and gram-negative bacteria, while TLR2 recognizes a variety of microbial components, such as peptidoglycan and lipoteichoic acids, from gram-positive bacteria (Abreu 2003, Matsuguchi et al., 2003, Takeda et al., 2003). Also, several other TLRs with specific actions are known, such as TLR5, which responds to the bacterial flagella (Rhee et al., 2005), and TLR9, which is activated by bacterially derived short DNA fragments containing CpG sequences (Pedersen et al., 2005). Other known recognition receptors are nucleotide-binding oligomerization domain proteins, which recognize both gram-positive and gram-negative bacteria. They are located in cell cytoplasm and are implicated in the induction of defensins. Increased epithelial barrier permeability is frequently associated with gastrointestinal disorders contributing to both disease onset and persistence (Lu and Walker 2001, Berkes 2003). The gatekeeper of the paracellular pathway is the tight junction, which is an apically located cell-cell junction between epithelial cells. The tight junction permits the passage of small molecules, such as ions, while restricting the movement of large molecules, such as antigens and microorganisms, which can cause inflammation. The integral membrane protein family, which are mainly claudins, occluding and zonula occludens 1, are implicated in the formation of the paracellular channels (Berkes et al., 2003).

6. Origine and safety of probiotics

An old dogma of probiotic selection has been that the probiotic strains should be of "human origin". One may argue that from evolutionary point of view, describing bacteria to be of human origin does not make much sense at all. The requirement for probiotics to be of human origin relates actually to the isolation of the strain rather than the "origin" itself. Usually, the strains claimed to be "of human origin" have been isolated from faecal samples of healthy human subjects, and have therefore been considered to be "part of normal healthy human gut microbiota". In reality the recovery of a strain from a faecal sample does not necessarily mean that this strain is part of the normal microbiota of this individual, since microbes passing the GI tract transiently can also be recovered from the faecal samples (Forssten et al., 2011). In practice it is impossible to know the actual origin of the probiotic strains, regardless of whether they have been isolated from faecal samples, fermented dairy

products or any other source for that matter. Isolation of a strain from faeces of a healthy individual is also not a guarantee of the safety of the strain—such a sample will also always contain commensal microbes which can act as opportunistic pathogens, or even low levels of true pathogens, which are present in the individual at sub-clinical levels. Therefore, it has been recommend that instead of concentrating on the first point of isolation, the selection processes for new potential probiotic strains should mainly focus on the functional properties of the probiotic strains rather than the "origin" (Forssten et al., 2011; Ouwehand and Lahtinen 2008).

As viable, probiotic bacteria have to be consumed in large quantities, over an extended period of time, to exert beneficial effects; the issue of the safety of these microorganisms is of primary concern (Leroy et al., 2008). Until now, reports of a harmful effect of these microbes to the host are rare. However, many species of the genera Lactobacillus, Leuconostoc, Pediococcus, Enterococcus, and Bifidobacterium were isolated frequently from various types of infective lesions. According to Gasser (1994), L. rhamnosus, L. acidophilus, L. plantarum, L. casei, Lactobacillus paracasei, Lactobacillus salivarius, Lactobacillus lactis, and Leuconostoc mesenteroides are some examples of probiotic bacteria isolated from bacterial endocarditis; L. rhamnosus, L. plantarum, Leuconostoc. mesenteroides, Pediococcus acidilactici, Bifidobacterium eriksonii, and Bifidobacterium adolescentis have been isolated from bloodstream infections and many have been isolated from local infections. Although minor side effects of the use of probiotics have been reported, infections with probiotic bacteria occur and invariably only in immunocompromised patients or those with intestinal bleeding (Leroy et al., 2008).

An issue of concern regarding the use of probiotics is the presence of chromosomal, transposon, or plasmid-located antibiotic resistance genes amongst the probiotic microorganisms. At this moment, insufficient information is available on situations in which these genetic elements could be mobilised, and it is not known if situations could arise where this would become a clinical problem (Leroy et al., 2008). When dealing with the selection of probiotic strains, the FAO/WHO Consultancy recommends that probiotic microorganisms should not harbor transmissible drug resistance genes encoding resistance to clinically used drugs (FAO/WHO, 2002). For the assessment of the safety of probiotic microorganisms and products, FAO/WHO has formulated guidelines, recommending that probiotic strains should be evaluated for a number of parameters, including antibiotic susceptibility patterns, toxin production, metabolic and haemolytic activities, and infectivity in immunocompromised animals (FAO/WHO, 2002). In vitro safety screenings of probiotics may include, among others, antibiotic resistance assays, screenings for virulence factors, resistance to host defence mechanisms and induction of haemolysis. Several different animal models have been utilized in the safety assessment of probiotics. These include models of immunodeficiency, endocarditis, colitis and liver injury. In some cases even acute toxicity of probiotics has been assessed. Last but not least, also clinical intervention trials have yielded evidence on the safety of probiotics for human consumption (Forssten et al., 2011).

7. Conclusion

The individual diversity of the intestinal microflora underscores the difficulty of identifying the entire human microbiota and poses barriers to this field of research. In addition, it is apparent that the actions of probiotics are species and strain specific. It is also apparent that even a single strain of probiotic may exert its actions via multiple, concomitant pathways. Probiotics have long been used as an alternative to traditional medicine with the goal of maintaining enteric homeostasis and preventing disease. However, the actual efficacy of this treatment in still debated. Clinical trials have shown that probiotic treatment can reduce the risk of some diseases, especially antibiotic-associated diarrhea, but conclusive evidence is impeded owing to the wide range of doses and strains of bacteria used. The mechanism of action is also an area of interest **(**Ohland and MacNaughton, 2010). Many studies, as discussed above, have shown that probiotics increase barrier function in terms of increased mucus, antimicrobial peptides, and sIgA production, competitive adherence for pathogens, and increased TJ integrity of epithelial cells. Current investigation into the mechanism of action of specific probiotics has focused on probiotic-induced changes in the innate immune functions involving TLRs and its downstream systems Like NF-κB, and other pathways (Yoon and Sun, 2011). Although the immunomodulatory effects of probiotics have been demonstrated in experimental animal models of allergy, autoimmunity, and IBD, information from clinical trials in humans is scarce. Furthermore, some studies suggest that probiotics could induce detrimental effects. Therefore, more research, especially in the form of well-designed clinical trials, is needed to evaluate the efficacy and safety of probiotics (Ezendam and Van Loveren, 2008). With evolving knowledge, efective probiotic therapy will be possible in the future.

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Phenotypic and Genotypic Characterization of Some Lactic Acid Bacteria Isolated from Bee Pollen: A Preliminary Study

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In the present work, five hundred and sixty-seven isolates of lactic acid bacteria were recovered from raw bee pollen grains. All isolates were screened for their antagonistic activity against both Gram-positive and Gram-negative pathogenic bacteria. Neutralized supernatants of 54 lactic acid bacteria (LAB) cultures from 216 active isolates inhibited the growth of indicator bacteria. They were phenotypically characterized, based on the fermentation of 39 carbohydrates. Using the simple matching coefficient and unweighted pair group algorithm with arithmetic averages (UPGMA), seven clusters with other two members were defined at the 79% similarity level. The following species were characterized: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and unidentified lactobacilli. Phenotypic characteristics of major and minor clusters were also identified. Partial sequencing of the 16S rRNA gene of representative isolates from each cluster was performed, and ten strains were assigned to seven species: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus ingluviei*, *Pediococcus pentosaceus*, *Lactobacillus acidipiscis* and *Weissella cibaria*. The molecular method used failed to determine the exact taxonomic status of BH0900 and AH3133.

Key words: lactic acid bacteria, numerical clustering, pollen, functional foods, UPGMA, 16S rRNA gene sequencing

Beehive products (honey, pollen, propolis and royal jelly) are natural functional foods that have gained increased attention in society [1]. Pollen, the male gametophyte of flowering plants, is a high-energy material collected by honeybees and other insects and stored as a food reserve. Pollen has been used traditionally by humans as a supplementary food and in alternative medical treatments. It has been used medically in prostatitis, bleeding stomach ulcers and some infectious diseases [2].

Because of its complex content, bee-pollen has a very important nutritional value in the human diet [3]. Since the middle of the last century, the bee-pollen microflora has been investigated [4–10]. However, little is known about the occurrence of lactic acid bacteria (LAB), and the roles they play, in pollens. Only a few reports are available in the literature considering this field. Occurrence of lactobacilli in pollens was reviewed by Gilliam [7]. In another study, Vásquez and Olofsson [11] identified lactobacilli isolated from pollen grains. These isolates were identified based on PCR and 16S rRNA gene sequencing. Other LAB representative genera (*Lactococcus*, *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Leuconostoc*) were recovered and identified based on phenotypic traits [4]. In the latter investigation, in vitro studies indicated that several strains inhibit Gram-positive and Gram-negative pathogenic bacteria. Additionally, several members of LAB are known to produce antibacterial substances such as organic acids and bacteriocins. Antagonism towards undesirable microorganisms is an important criterion for LAB being used as bio-preservations or biocontrol agents. It seems that pollens are a suitable ecological niche for various microorganisms and an important source for the isolation of new strains belonging to the LAB group with

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Table 1. Indicator bacteria used and their growth conditions

Indicator species	Strain no.	Origin	Growth medium	Incubation conditions
Gram-positive bacteria				
Bacillus subtilis	20302	CLAM[a]	BHI[c]	37°C
Enterococcus faecium	H421	CLAM	MRS[d]	30°C
Listeria innocua	3030	CLAM	BHI	37°C
Staphylococcus aureus	25923	ATCC[b]	BHI	37°C
Gram-negative bacteria				
Escherichia coli	25922	ATCC	BHI	37°C
Salmonella typhimurium	1717	CLAM	BHI	37°C
Pseudomonas aeruginosa	27853	ATCC	BHI	37°C
Shigella sp.	96415	CLAM	BHI	37°C

[a] Collection of Laboratory for Applied Microbiology
[b] American Type Culture Collection
[c] Brain heart infusion broth
[d] De Man, Rogosa and Sharpe broth

antagonistic activity against harmful bacteria. Species or subspecies identification of such strains is recommended. In fact, physiological and biochemical criteria used for LAB identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, clear identification to the species level by simple phenotypic tests may sometimes be difficult [12]. Indeed, additional molecular-based characterization approaches such as 16S rRNA gene sequencing, 16S-ARDRA, PFGE and other methods are used for assignment of a given LAB strain to its taxonomic status.

The present work describes isolation and phenotype-based numerical clustering of LAB isolated from pollen grains collected in some Algerian areas; 16S rRNA gene sequencing-based characterization of selected strains was investigated. Also, their antagonistic activity against Gram-positive and Gram-negative bacteria was evaluated.

EXPERIMENTAL

Isolation of LAB from pollen

Previous studies [4] indicate that pollen samples taken from pollen traps are highly contaminated, especially by enterococci. Furthermore, dilution-based LAB isolation is not a sufficient manner for the recovery of these bacteria from pollen grains, and full negative results are unavoidable with this method. Hence, a simple technique is used for the efficient isolation of LAB. In the field during flowering seasons, honey bee foragers are kinched (catched) by a sterile stainless steel or plastic forceps. The pollen pellets (60 samples from 10 clean regions; six subsamples from each sampling site; in Bordj Bou Arreridj and Sétif) are then collected by a humidified sterile cotton swab by gently touching the pollen pellets attached to the posterior legs of the bee. At this time, we can discuss the facts regarding bee-collected pollen from a microbiological point of view. The swabs were transported to the laboratory at low temperature (5°C) and analyzed or further maintained with refrigeration until use. Because LAB isolation is enrichment-based method, the quantity of pollen recovered is not important. Each swab was then introduced into a capped glass tube containing 15 mL Elliker broth pH 6.5 [13], and incubated anaerobically at 30°C for at least two days. Afterwards, serial dilutions were prepared from each tube in peptone water (0.1% peptone and 0.1% of Tween 80), and from the appropriate dilution, aliquots (100 µL) were spread and cultured on the following media: M17 agar (Fluka) incubated for 72 hr at 30°C for lactococci and streptococci, LBS agar [14] after anaerobic incubation (BBL GasPack System) at 30°C for 72 hr for lactobacilli, Glucose Yeast Extract Agar [15] incubated at 30°C for 48 hr to isolate leuconostocs and pediococci, and D-MRS agar [16] incubated at 25°C for 72 hr to isolate carnobacteria. Enterococci were treated in a separate study. From each culture, 10–30 colonies were randomly picked up and further purified on MRS agar (pH 6.5). Pure isolates were maintained at –20°C in MRS broth containing glycerol (20%, v/v final concentration).

Screening for antagonistic activity

For detection of antagonistic activity, an agar well diffusion assay was used according to Schillinger and Lucke [17]. Bacterial species used as indicator

microorganisms were listed in Table 1. LAB isolates were subcultured twice (1% inoculums, 24 hr, 30°C) in 10 mL MRS broth (Fluka). The non-LAB were subcultured twice (1% inoculum, 24 hr, 37°C) in 10 mL BHI broth (Fluka) and kept frozen at –20°C in BHI broth supplemented with 20% glycerol. Cell-free supernatants from LAB cultures were obtained by centrifuging the cultures (8,000 g/10 min at 4°C), and then the pH of each supernatant was adjusted to 6.5 using 5N HCl followed by filtration through a cellulose acetate filter with a pore size of 0.2 µm. Before supernatant neutralization, an antimicrobial assay was performed for all isolates. All experiments were performed in duplicate, and the results were displayed as the mean value of the experiments. Isolates showing antagonistic activity against one or more indicator bacteria (with an inhibition zone diameter of more than 5 mm) were subjected for phenotypic characterization.

Phenotypic identification of bioactive isolates

Isolates exhibiting antagonistic activity were selected on the basis of Gram staining, morphology, tetrad formation and catalase activity. Catalase-negative and Gram-positive rods and cocci were selected and screened for the production of CO_2 from glucose (in MRS broth, containing Durham inverted tubes, without beef extract and citrate). Ability to grow at 10 and at 45°C was evaluated in MRS broth after incubation for 7 days and 48 hr, respectively. Growth in MRS containing 6.5 or 18% NaCl, as well as growth in MRS with pH 4.4 and 9.6, was studied. Acid production from carbohydrates (glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D-arabitol, L-arabitol, and gluconate) was evaluated by miniaturized assay in 96-well flat-bottom microtiter plates [18]. Sterile carbohydrate solutions (Institut Pasteur, France) were added to the basal medium (MRS without glucose and meat extract and with 0.16 g/L bromocresol purple, pH 7.0) at a final concentration of 1%. Cells were harvested from overnight cultures by centrifugation (10,000 g, 5 min, 4°C), washed twice in sterile phosphate buffer (pH 7.0) and suspended in sterile saline (0.85% NaCl). This suspension was used to inoculate sterile microtiter plates (96 flat-bottom wells), which were incubated in anaerobiosis for 7 days at 30°C. Esculin hydrolysis was assessed by adding 2 g/L esculin (Sigma) and 5 g/L ferric ammonium citrate (Sigma) to the basal medium.

Fermentation of each of the 39 carbohydrates was interpreted as follows: positive (+), complete change to yellow; weakly positive (w), change to green; and negative (-), no change at all. Esculin hydrolysis (revealed by a change to a darker color) was interpreted as positive (+), while no change was negative (-). Strains were tested in duplicate to determine the test reproducibility.

Genotypic identification of selected LAB strains

Sample preparation prior to PCR amplification

As described by Rodas et al. [19], selected LAB isolates (10 strains displaying a remarkable assimilatory pattern) were grown in MRS agar at 30°C for 2 days. One single colony was picked up from plates and suspended in 20 µL of sterile distilled water. These suspensions were used for PCR reactions without further processing.

Amplification and sequencing of 16S rRNA gene

The protocol of Rodas et al. [19] was used for 16S rRNA gene amplification using primers pA (8-AGAGTTTGATCCTGGCTCAG-28) and pH (1542-AAGAGGTGATCCAGCCGCA-1522) [20]. DNA amplification was carried out in a 50 µL PCR mixture containing 200 µM dNTP, 1 µM of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 6 U of Taq DNA polymerase and 1 µL of the cell suspension. Each PCR cycle consisted of an initial denaturation time of 5 min at 94°C followed by 35 cycles of amplification comprising a denaturation step for 30 sec at 94°C, annealing at 56°C for 30 sec, and extension at 72°C for 1 min. Reactions were completed with 5 min elongation at 72°C followed by cooling to 10°C. PCR products were resolved by electrophoresis at a constant voltage (200 V) in 1.2% (w/v) agarose in 0.5 x TBE (45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA pH 8.0), gels were stained with ethidium bromide (0.5 µg/mL). Amplification products were purified using a PCR purification kit (Qiagen, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and an automated DNA sequencer (ABI Prism® 3100 Avant Genetic Analyzer, Applied Biosystems). The nucleotide sequences of the 16S rRNA gene of all the isolates were analyzed and determined by the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov/). The alignments were analyzed to construct a phylogenetic tree and to compare similarities among the sequences by the neighbor-joining method [21] using MEGA software version 4.0. Bootstrap analysis was used to evaluate the tree topology of the data by performing 1,000 resamplings. The sequences were deposited in the GenBank database using the web-based data submission

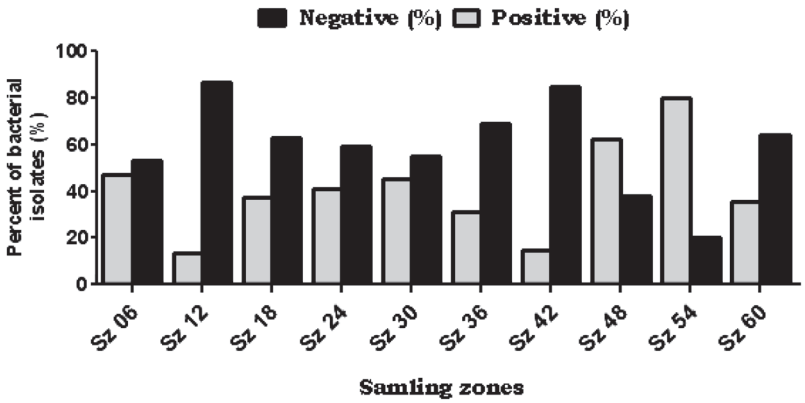

Fig. 1. Percent distribution of antagonism among 567 LAB strains isolated from pollen samples. Samples Sz06, Sz18, Sz30, Sz36 and Sz54 were from BBA, whereas samples Sz12, Sz24, Sz42, Sz48 and Sz60 were from Sétif. Statistical analysis indicates that the total percentage of potent strains (40.74 ± 6.31) is significantly different ($p=0.003 < 0.05$) from that of non-potent strains (59.26 ± 6.31). Percent distributions of potent strains were extremely different from each other ($p<0.0001$) except for sampling zones Sz36 and Sz60 ($p<0.01$). Also, there were no statistical differences between the percentage of potent strains of sampling zones Sz06 and Sz30 ($p>0.05$) or sampling zones Sz18 and Sz60 ($p>0.05$). Percent distributions of non-potent strains among sampling zones were significantly different from each other ($p<0.05$) except for sampling zones Sz06 and Sz30, Sz12 and Sz42 and Sz18 and Sz24 and Sz18 and Sz60, which do not differ statistically ($p>0.05$).

tool Sequin (http://www.ncbi.nlm.nih.gov/Sequin).

Statistical analysis

Hierarchical cluster analysis was carried out with Statistica 6 software (Statsoft Italia, Padua, Italy). The Euclidean distance, unweighted pair group method with arithmetic mean (UPGMA) and an index of similarity were used for the analysis of carbohydrate fermentation. A two-way ANOVA test was used for comparing antimicrobial activity of LAB.

RESULTS

Isolation and antagonism among LAB strains

From the ten sampling zones, distributed across two provinces, six samples were collected from each zone. However, a total of 567 isolates were recovered from pollen grains. Growth of yeasts and molds on isolation agar media was observed and confirmed by microscopic examination. Also, catalase-positive bacterial colonies were encountered. Presumptive LAB cells were carefully selected based on catalase reaction, cell shape, motility and Gram staining. All Gram-positive, catalase-negative cocci and rods were purified on suitable agar media to homogeneity. The 567 pure LAB strains were screened for their antagonistic activity by agar well diffusion assay against eight undesirable bacteria belonging to Gram-positive and Gram-negative ranks (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CLAM 20302, *Enterococcus faecium* CLAM H421, *Listeria innocua* CLAM 3030, *Shigella* sp. CLAM 96415, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CLAM 1717, and *Pseudomonas aeruginosa* ATCC 27853). After a random screening process, using neutralized supernatant cultures, 216 stains found to potentially inhibit at least one indicator bacteria, whereas 351 were not. Statistical analysis indicated that total percentage of potent strains (40.74 ± 6.31) was significantly different ($p=0.003 < 0.05$) from that of non-potent strains (59.26 ± 6.31). From all sampling zones, except zones Sz48 (62.06%) and Sz54 (80.00%), the percentage of antagonistic LAB strains was below 46 (Fig. 1).

The 216 LAB strains showing inhibition zones against at least one indicator bacterium (without pH neutralization of spent cultures), according to the first antimicrobial screening assay, were further subjected to determination of the antibacterial activity of neutralized cell-free supernatants obtained by centrifugation. Neutralization of culture supernatant pH (6.5) eliminates the effect of acidity on the target bacteria. Furthermore, incubation of LAB under anaerobic conditions minimizes hydrogen peroxide production. Following the second screening assay using eight indicator bacteria (four Gram negative

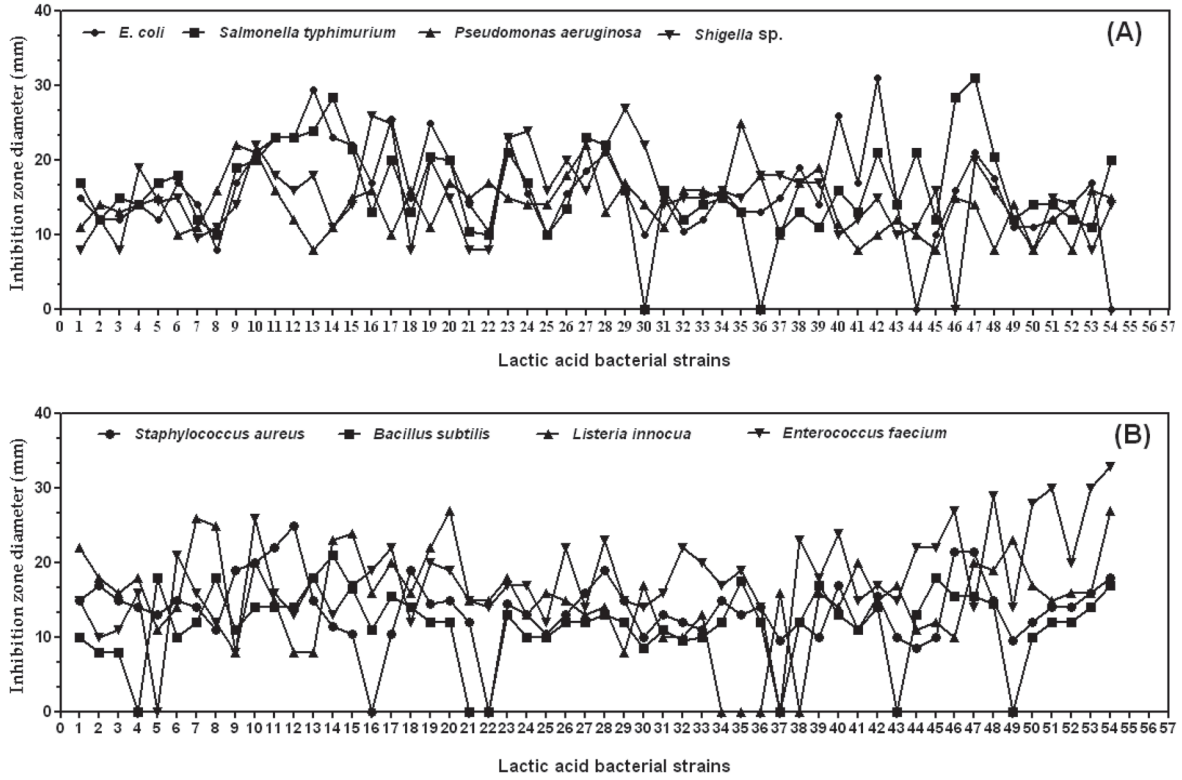

Fig. 2. Antibacterial activity (inhibition zone diameter, mm) of 54 pollen residing lactic acid bacterial strains against Gram-negative and Gram positive indicator bacteria (A) *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CLAM 1717, *Pseudomonas aeruginosa* ATCC 27853 and *Shigella* sp. CLAM 96415. Significant statistical differences were observed ($p=0.0071$, $p<0.05$) among antimicrobial potential of LAB strains, whereas indicator pathogenic bacteria themselves could not affect the variation in antimicrobial activity ($p=0.1033$, $p>0.05$) as determined by two-way ANOVA test. (B) *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* CLAM 20302, *Enterococcus faecium* CLAM H421, and *Listeria innocua* CLAM 3030. Two-way ANOVA analysis indicated that there were no significant statistical differences among the antimicrobial actions of LAB strains ($p>0.05$), but is resulted from the indicator pathogenic bacteria themselves and extremely significant differences were obtained ($p<0.0001$).

and four Gram positive), 54 potent LAB strains were obtained (Fig. 2). Figure 2A. presents the variation in the antagonistic activity against Gram-negative human and animal pathogenic bacteria. The activity was measured from the zone of inhibition (mm) around the well. This quantified activity varied from 10 to 27 mm for the majority of LAB strains studied (Fig. 2). Furthermore, only two strains (strains 43 and 54) could not inhibit *E. coli* ATCC 25922, two other strains (strains 30 and 36) were inactive against *Salmonella typhimurium* CLAM 1717, and strain 46 was inactive against *Shigella* sp. CLAM 96415 (Fig. 2A). In addition, only five strains were able to produce a zone of inhibition in between 29 and 35 mm against indicator strains, for example, strains 12 and 41 against *E. coli* ATCC 25922 and strains 14, 46 and 47 against *Salmonella typhimurium* CLAM 1717 (Fig. 2A). However, it was observed that *Pseudomonas aeruginosa* ATCC 27853 resisted the inhibitory potential as compared with the other indicator bacteria (Fig. 2A).

For Gram-positive indicator bacteria, the majority of inhibition diameters were between 10 and 20 mm for *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* CLAM 20302 and between 10 and 30 mm for *Enterococcus faecium* CLAM H421 and *Listeria innocua* CLAM 3030 (Fig. 2B). However, 14 strains were inactive against indicator bacteria. Strains 5, 21, 22, 37, 43 and 49 were inactive against *Bacillus subtilis*; strains 16 and 22 were inactive against *Staphylococcus aureus*; strains 34, 35, 36 and 38 were inactive against *Listeria innocua*; and strains 5 and 37 were inactive against *Enterococcus faecium*. In addition, it was noted that *Listeria innocua* and *Enterococcus faecium* were more susceptible to the action of LAB strains than *Bacillus subtilis* and *Staphylococcus aureus*. For the latter bacterium, the

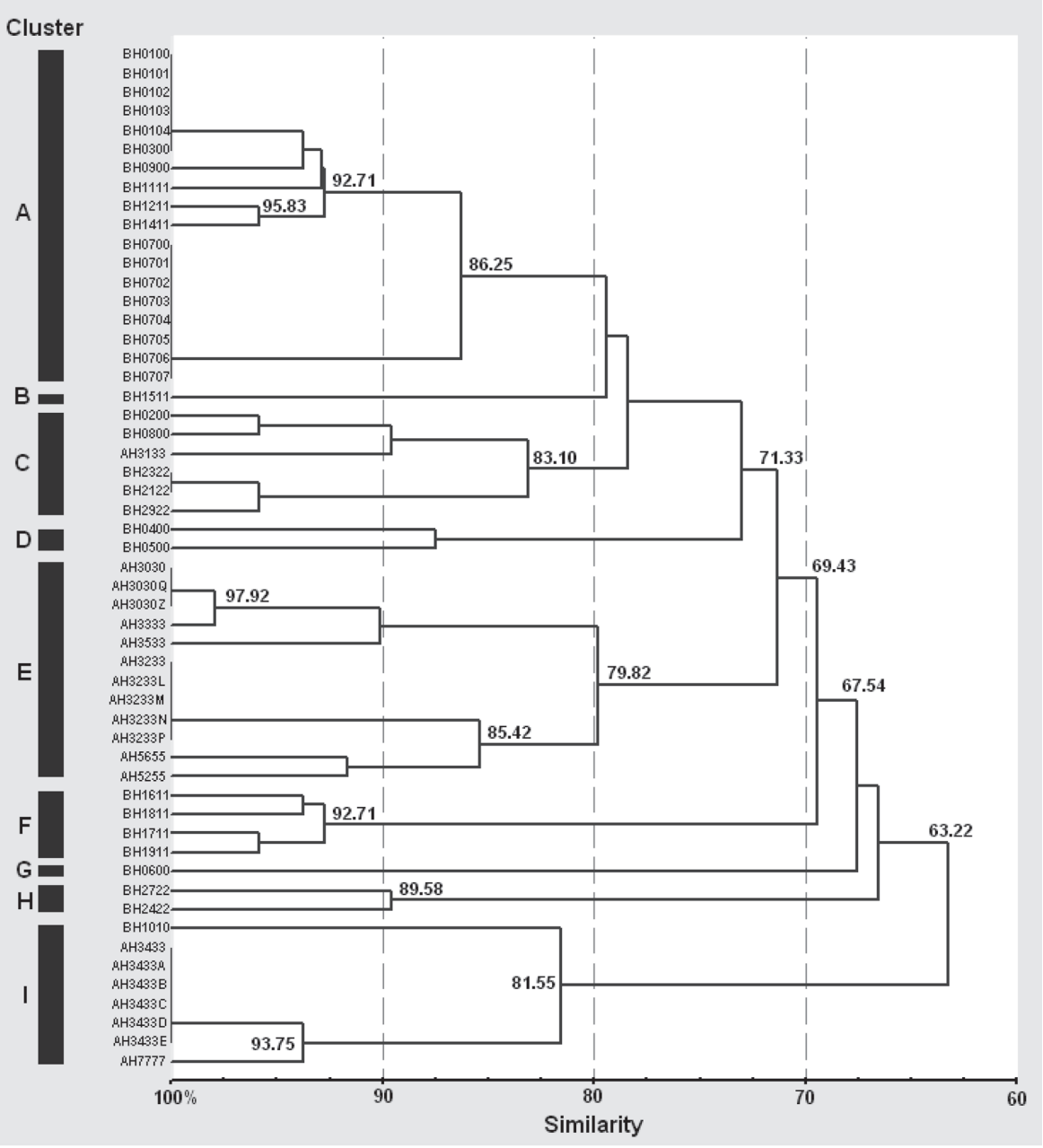

Fig. 3. Dendrogram of combined phenotype profiles of 54 potent antagonistic lactic acid bacteria isolates as determined by carbohydrate fermentation and physiologic traits. Cluster analysis was carried out based on the simple matching coefficient and unweighted pair group algorithm with arithmetic averages (UPGMA). Codes of strains and clusters are indicated on the left hand side of the figure.

inhibition diameters were between 10 and 33 mm (Fig. 2B).

Clustering structure and analysis

Fifty-four bacteriocin-like producing isolates of LAB obtained from different raw pollen samples were characterized according to the method of Axelsson [22], Bergey's Manual [23] and the prokaryotes [24]. Preliminary, the diversity was studied based on a phenotypic approach. Microplates containing 39 different carbon sources and 10 other physiological traits were used to determine the phenotypic profiles of the 54 isolates of

LAB. The reproducibility of the fermentation tests was 100%. The similarity coefficient cluster analysis resulted in five major clusters (A, C, E, F and I, containing three or more strains) defined at the 79.0% similarity level (Fig. 3). The following four other minor clusters were defined at the 79% similarity level (B, D, G and H, two 2-member cluster and two 1-member clusters).

Major clusters (A, C, E, F and I)

Cluster A), *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus* sp., comprised 18 isolates from Bordj Bou Arreridj (BBA) (33.33% of the total isolates). The cluster contained eight other *Lactobacillus plantarum* strains, one strain of *Lactobacillus paraplantarum* and eight *Lactobacillus* sp. strains. All strains were homofermentative, and 55.5% of the isolates in this cluster fermented L-arabinose. They did not ferment xylose, adonitol, β-methyl-xyloside, starch, arabitol and rhamnose. Alpha-methyl-D-mannoside was variously fermented. The same pattern was noted for gluconate, lactose and sorbitol. Members of this cluster grew at 10 and 45°C in the presence of 6.5% NaCl and at pH 4.4.

Cluster C), *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus* sp., contained three *Lactobacillus* sp. members, one strain of *Lactococcus lactis* subsp. *lactis* and two *Lactobacillus plantarum* strains. All were homofermentative and grew well at 10 and 45°C, in the presence of 6.5% NaCl and under alkaline conditions (pH, 9.6). They all fermented ribose, hexoses, cellobiose, lactose, saccharose, trehalose and B-gentiobiose, but they did not ferment erythritol, D-arabinose, xylose, adonitol, β-methyl-xyloside, dulcitol, α-methyl-D-glucoside, starch, xylitol, L-arabitol, D-lyxose, D-tagatose, D-fucose and L-fucose. They variously fermented L-arabinose, rhamnose, inositol, mannitol, sorbitol, α-methyl-D-mannoside, melibiose, melezitose, D-raffinose, D-arabitol, gluconate and amygdalin.

Cluster E), *Lactococcus lactis*, *Pediococcus pentsaceus* and *Pediococcus acidilactici*, contained five strains of *Lactococcus lactis* subsp. *lactis* (AH3030, AH3031, AH3032, AH3333 and AH3533), six strains of *Pediococcus pentosaceus* (AH3233, AH3233 L, AH3233 M, AH3233 N, AH3233 and AH5655), and one strain of *Pediococcus acidilactici* (AH5255). All strains of this group were homofermenters and grew well at 45°C. The strains AH3030, AH3031, AH3032 and AH3333 did not grow at 10°C or in the presence of 6.5% sodium chloride. They did not ferment D-arabinose, β-methyl-xyloside, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose and melezitose, but they did ferment ribose, galactose, D-glucose, D-fructose, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin and cellobiose. Furthermore, L-arabinose, D-xylose, D-mannose, mannitol, lactose, B-gentiobiose, maltose and saccharose were variously fermented. In addition, only the strain, *Lactococcus lactis* AH3533, was able to ferment starch and gluconate.

Cluster F), this cluster contained four strains of *Lactobacillus* sp., BH1611, BH1711, BH181 and BH1911. They grew at pH 4.4, at 10 and 45°C and in the presence of 6.5% sodium chloride, but they did not grow at pH 9.6. They fermented ribose, xylose, hexoses, α-methyl-D-mannoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose and D-raffinose. They did not ferment melezitose, gluconate, inositol, mannitol, rhamnose, D-arabinose and β-methyl-xyloside.

Cluster I), contained strains of *Pediococcus pentosaceus* and *Lactobacillus plantarum*. Members of this cluster (seven strains of *Pediococcus pentosaceus* and one strain *Lactobacillus plantarum* BH1010) were homofermenters. All strains were able to grew at 10 and 45°C, at pH 4.4 and in the presence of 6.5% NaCl. However, D-arabinose, L-arabinose, β-methyl-xyloside, galactose, D-glucose, D-fructose, α-methyl-D-glucoside, N-acetyl glucosamine, amygdalin, arbutin, salicin, saccharose, xylitol and D-lyxose were fermented by members of this group. Adonitol was fermented by all strains except *Pediococcus pentosaceus* AH7777 and *Lactobacillus plantarum* BH1010. In addition, none of the strains fermented ribose, xylose, D-mannose, L-sorbose, rhamnose, dulcitol, sorbitol, α-methyl-D-mannoside, trehalose, D-raffinose, starch, gentiobiose, gluconate and D-arabitol. Melezitose, inositol, mannitol, maltose and lactose were fermented only by *Lactobacillus plantarum* BH1010.

Minor clusters and stragglers (B, D, G and H)

Cluster B contained one strain of *Lactobacillus fermentum*; cluster D contained two strains, *Lactobacillus fermentum* and *Lactobacillus* sp. One strain identified as *Lactobacillus plantarum* was assigned to cluster G. However, two other strains, *L. fermentum*, were grouped in cluster H. All strains were heterofermenters and grow at 45°C and in the presence of 6.5% of sodium chloride with the exception of strain BH2422. Members of cluster H were able to grow at pH 9.6 compared with strains of the other clusters. None of the strains fermented erythritol, adonitol, β-methyl-xyloside, D-lyxose,

Table 2. Phenotypic profiles of the 54 potent LAB isolates as determined by carbohydrate fermentation

	Cluster A 18 isolates	Cluster B (BH1511)	Cluster C 6 isolates	Cluster D (BH0400 BH0500)	Cluster E 12 isolates	Cluster F 4 isolates	Cluster G (BH0600)	Cluster H (BH2722 BH2422)	Cluster I 8 isolates
D-Arabinose	5.5	0	0	0	0	0	0	50	100
L-Arabinose	55.5	100	33.33	100	41.66	0	0	100	100
Ribose	44.5	100	100	50	100	100	100	100	0
Adonitol	0	0	0	0	0	0	0	0	75
β-M-xyloside	0	0	0	0	0	0	0	0	100
D-Mannose	100	0	100	50	58.33	100	0	0	0
Rhamnose	0	100	50	0	0	0	0	50	0
Inositol	0	0	33.33	50	0	0	0	0	12.5
Mannitol	100	100	83.33	50	41.66	0	0	50	12.5
Sorbitol	50	100	83.33	0	0	100	0	50	0
Alpha-M-D-mannoside	38.88	0	50	50	0	100	0	50	0
β-M-xyloside	0	0	16.2	50	0	50	0	0	100
Cellobiose	100	0	100	50	100	100	0	50	25
Maltose	100	100	100	50	91.66	100	100	100	12.5
Lactose	10	100	100	100	41.66	100	100	100	12.5
Melibiose	100	100	66.7	50	0	100	100	100	87.5
Saccharose	100	100	100	100	41.66	100	100	100	100
Trehalose	100	0	100	50	91.66	100	0	0	0
Melezitose	100	100	83.33	50	0	0	0	50	12.5
D-Raffinose	94.5	100	50	0	0	100	100	100	0
Starch	0	0	0	0	8.33	75	0	50	0
Gentiobiose	100	100	100	50	91.66	0	0	100	0

Numbers indicate the percent positive reaction of each test for the isolates of each cluster.

D-tagatose, D-fucose, L-arabitol and 2-keto-gluconate. However, strain BH2422 (cluster H) was also unable to ferment D-arabinose. Table 2 highlights the different carbohydrates variously fermented by members of the clusters obtained at the similarity level of 79% (A-I).

Genotypic characterization of selected LAB strains

Sequences of the 16S rRNA gene (approximately 1,500 bp) of ten LAB isolates, BH0900, BH1511, AH3133, BH0500, AH5655, AH3030, BH1711, BH0600, BH2422, and AH3433A, were determined. The 16S rRNA nucleotide sequences of the isolates were aligned with homologous regions from various LAB, and a phylogenetic tree was constructed by the neighbor-joining method (Fig. 4). The BLAST analysis of 16S rRNA gene sequences of the selected strains showed alignments of these sequences with reported 16S rRNA genes in the gene bank. The nucleotide sequences were deposited in GenBank, and accession numbers for strains BH0900, BH1511, AH3133, BH0500, AH5655, AH3030, BH1711, BH0600, BH2422 and AH3433A were obtained (KF178303, KF178304, KF178305, KF178307, KF178308, KF178306, KF178310, KF178311, KF178312 and KF178309, respectively). However, the 10 isolates were assigned to seven species, *Lactobacillus plantarum*, *L. fermentum*, *Lactococcus lactis*, *L. ingluviei*, *Pediococcus pentosaceus*, *L. acidipiscis* and *Weissella cibaria*.

On the basis of phylogenetic data obtained, strain BH0900 showed similarity (99%) with *Lactobacillus plantarum* WCFS1 (075041.1) and *Lactobacillus pentosus* 124-2 (029133.1) as well as with *Lactobacillus paraplantarum* DSM 10667 (025447.1) and *Lactobacillus plantarum* NRRL B-14768 (042394.1). However, strain BH1511 shares 99% similarity with *Lactobacillus fermentum* IFO 3956 (075033.1) and 96% similarity with *Lactobacillus gastricus* Kx156A7 (029084.1). On the other hand, strains AH3133 and AH3030 share 99% similarity with *Lactococcus lactis* subsp. *lactis* NCDO 604 (040955.1), *Lactococcus lactis* subsp. hordniae NCDO 2181 (040956.1), *Lactococcus lactis* subsp. *cremoris* SK11 (074949.1), *Lactococcus lactis* subsp. *cremoris* NCDO 607 (040954.1) and 92% similarity with *Lactococcus plantarum* DSM 20686 (044358.1). Furthermore, 99% similarity was shared between isolate BH0500 and *Lactobacillus ingluviei* KR3 (028810.1). In addition, two strains, AH5655 and AH3433A, were closely related (99%) to *Pediococcus pentosaceus* DSM 20336 (042058.1) and showed more than 98% similarity to *Pediococcus stilesii* LMG 23082 (042401.1). One strain,

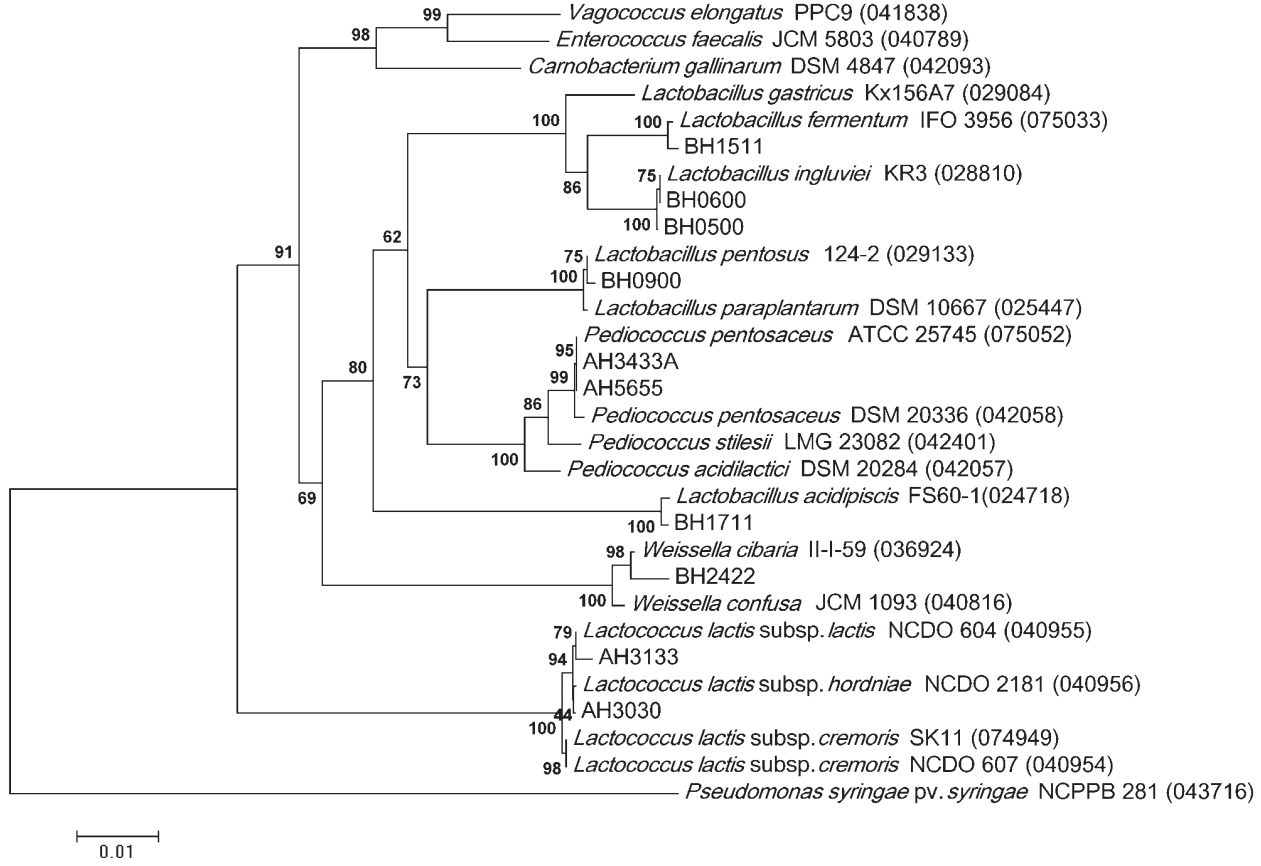

Fig. 4. Phylogenetic tree based on 16S rDNA sequence analysis, showing the phylogenetic placement of selected LAB strains isolated from pollen grains. The tree was constructed by the neighbor-joining method, and *Pseudomonas syringae* pv. *syringae* was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

BH1711, displayed 98% similarity with *Lactobacillus acidipiscis* FS60-1 (024718.1). Strain BH0600, which was phenotypically identified as *Lactobacillus plantarum*, was genotypically related (99%) to *Lactobacillus ingluviei* KR3 (028810.1). Finally, strain BH2422 was considered *Lactobacillus fermentum* based on phenotypic characterization; however, 16S rRNA gene sequence analysis revealed that this strain was related to *Weissella* and shared 99% sequence similarity with *Weissella cibaria* II-I-59 (036924.1).

DISCUSSION

Isolation of LAB and antagonistic activity

The effectiveness of LAB is a strain-dependant aspect. This trait may be obtained by genetic manipulation or, as is frequently the case, searching for new desirable strains in natural niches. Plants, foods, fermented products, animals and humans constitute natural ecological systems and good sources for LAB. To the best of our knowledge, antagonism of LAB against human bacterial gastrointestinal pathogens is the most important feature for selecting such a strain designed for the human gut.

Here, we described isolation of LAB from raw pollen grains showing antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria. The 54 LAB effective strains were characterized by means of phenotypic tests. The relationships among the phenotypically characterized strains of LAB were determined by cluster analysis. It is well known that pollen grains are sterile before anther opening. Flowers and their constituents are parts of the plant phylloplane. Stirling and Whittenburg [25] suggested that the LAB are not usually part of the normal microflora of the growing plant and indicated the role of insects in the spread of these organisms. LAB commonly found on fresh herbage and in silage have been investigated [26, 27]. Nilsson and Nilsson [28] were found that the predominant

LAB during silage fermentation were streptococci and lactobacilli, with *Lactobacillus plantarum* the species most frequently recovered. Other studies [29, 30] reported the occurrence of pediococci and lactobacilli on leaving or decayed plants. Lactobacilli commonly share the habitat phyllosphere with species of the genera *Leuconostoc*, *Pediococcus* and *Weissella*. Species frequently recovered from the leaves include *L. plantarum*, *L. paracasei*, *L. fermentum*, *L. brevis* and *L. buchneri* [16], which is in line with our results. Part of the accumulated information about the occurrence of lactobacilli (and other LAB members) on plants is derived from microbiological studies of the fermentation process. Thus, the microbial population upon initiation of the process is known for several plants (grasses, cabbage, silage raw materials, carrots and beets, olives and fruits such as grapes and pears, etc.). But scarce information about the occurrence of LAB on flowers and pollens is available in the literature. Indeed, we report in this work the occurrence of LAB in pollen grains and for the first time the isolation and characterization of several species belonging to LAB. The results reported here indicate that 54 isolates (25%) of the total antagonistic isolates (216 strains) inhibit indicator bacteria. Inhibition caused by hydrogen peroxide and organic acids was ruled out, as the producer strains were cultured anaerobically and the culture supernatant was neutralized (pH 6.5) before assaying the antimicrobial activity. This study indicates that the compound inhibiting the microbial growth in the neutralized cell-free supernatant was not organic acid or hydrogen peroxide commonly produced by many LAB [31].

As reported by Daeschel et al. [32], certain LAB protect plants by producing antagonistic compounds [12] contributing to inhibition of the plant pathogens *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae*. Furthermore, LAB are well known for their antagonism towards other Gram-positive bacteria, especially taxonomically related species (*Listeria* spp., *Bacillus* spp. *Micrococcus* spp., etc.), and Gram-negative plant and animal pathogens, such as *Escherichia coli*, *Salmonella* spp., *Helicobacter pylori* and *Pseudomonas aeruginosa*. Contrary to what is believed, that LAB are more potent in inhibiting Gram-positive bacteria than Gram-negatives bacteria, which is claimed for the type of cell wall of the target microorganism, this study reveals that Gram-negative bacteria used in this study (*E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Shigella* sp.) were susceptible to cell-free supernatants from tested LAB, especially the strains 9 (*Pediococcus pentosaceus* AH3433E), 12 (*Lactobacillus plantarum* BH1010), 14 (*Lactobacillus plantarum* BH0600), 16 (*Lactobacillus* sp. BH1811), 19 (*Pediococcus acidilactici* AH5255), 29 (*Lactobacillus plantarum* BH0800), 39 (*Lactobacillus plantarum* BH1411), 41 (*Lactobacillus plantarum* BH0104) and 47 (*Lactobacillus plantarum* BH0102). These results are in accordance with earlier results reported by Trias et al. [33], who showed that most LAB originating from fruits and vegetables displayed good antagonistic activity against foodborne pathogens, such as, *Listeria monocytogenes*, *Salmonella typhimurium* and *Escherichia coli*. Several indicator strains displayed different degrees of susceptibility towards antimicrobial compounds from a given producer strain; for example, strain 46 (*Lactobacillus* sp. BH2122) inhibits strongly *E. coli*, *Salmonella typhimurium* and moderately inhibits *Pseudomonas aeruginosa* but is inactive towards *Shigella* sp. The indicator strain *L. innocua* was used in this study instead of *L. monocytogenes*, as the two microorganisms show similar physiological properties with the difference that the former does not belong to the pathogenic species of *Listeria*. Moreover, some papers have reported a greater sensitivity of *L. monocytogenes* towards some antibacterial compounds than *L. innocua* [34, 35].

It is well known that the presence of lactobacilli is important for maintenance of the intestinal microbial ecosystem [36]. They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes* [37, 38], *Escherichia coli* and *Salmonella* spp. [39, 40]. This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, and bacteriocins. Our results agreed with the latter statements; therefore, our isolates are strong candidates for clinical use during gut treatment regimes. Furthermore, a major advantage of using LAB as biocontrol agents is that they are considered GRAS (generally recognized as safe) and usually comply with all recommendations for food and drug products [41]. Moreover, LAB are natural colonizers of fresh plant products and have been previously described as good antagonists of several bacteria and fungi [42, 43].

Siezen et al. [44] hypothesized that the fermentative profile reflects the original habitat and that lactose utilization is less prevalent in plant isolates with respect to those from cheese and the human gastrointestinal tract. Indeed, lactose was fermented by most isolates in the present study, except isolates of *Pediococcus* from clusters I and E and isolates of *Lactobacillus* sp. from cluster A. The inability of plant-related LAB to ferment lactose was presumably due to the relatively recent acquisitions, via horizontal gene transfer and subsequent

natural selection, of lactose metabolic genes, which are often plasmid encoded in dairy and human strains [45]. Contrary to the findings of Cagno et al. [46], who studied *Lactobacillus plantarum* from vegetables and fruits, all *Lactobacillus plantarum* isolates of this study used this carbon source.

Overall, all isolates fermented maltose and cellobiose except *Pediococcus acidilactici* AH5255 (cluster E) and *Pediococcus pentosaceus* AH3433, AH3433A, AH3433B, AH3433C, AH3433D and AH3433E (cluster I). Strain AH7777 (*Pediococcus pentosaceus*; cluster I) fermented cellobiose but not maltose. Also, cellobiose was not fermented by *Lactobacillus fermentum* BH0400, *Lactobacillus* sp. BH0500 (cluster D), members of cluster H (*Lactobacillus fermentum* BH2722, and BH2422) and members of clusters B and G. Arabinose, glucose, fructose, mannose, mannitol, B-gentiobiose, melezitose, melibiose, saccharose and trehalose were variously fermented. These carbon sources correspond mainly to those prevalent in the plant kingdom [47]. Similar phenotypic profiles were found for *Lactobacillus plantarum* isolated from Thai fermented fruits and vegetables [45, 47, 48]. Glycosides such as amygdalin were not used only by isolates of clusters H, D, B and C, salicin was not used only by eight isolates belonging to cluster A, (*Lactobacillus* sp. BH0700, 0701, 0702, 0703, 0704, 0705, 0706 and 0707). On the other hand, arbutin was used by isolates of all clusters. These glycosides are typically found in vegetables.

Starch is a reserve polysaccharide in pollen grains, and starch-hydrolyzing strains belong to the clusters E, F, and H. According to the fermentation profile, it seems that the isolates assimilate variously a panel of carbohydrates that reflects their enzymatic and genetic potentials. Furthermore, these traits were shared with LAB of dairy or animal origin. Based on the limited number of tests used, phenotypic profiles did not cluster the isolates according to the original habitat. This was probably because the studied isolates were obtained from raw pollen samples having a very similar chemical composition. It could also have been because the pollen residing LAB were of animal as well as plant origin. Nevertheless, phenotypic profiling was useful to understand the manifestations of environmental adaptation, which will be reflected in the technological processes.

From the different phenotypic clusters, ten selected isolates (displaying a good assimilation activity) were identified by means of 16S rRNA gene sequencing. It is well known that the species *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes. In the present results, misidentification of some isolates to defined species was encountered. More molecular techniques should be used for determination of the taxonomic status of these strains. The occurrence of *Weissella cibaria*, *Lactobacillus ingluviei* and *Lactobacillus acidipiscis* in pollen grains is in accordance with the ubiquity of these bacteria in nature [24]. In fact, insects, honeybees, soil, water and animal and human feces are main microbial contamination sources of pollens [4, 5]. From another point of view, misidentification of some isolates by phenotypic traits is probably due in part to the limited characters used for this purpose, or by the similarity of the metabolic patterns expressed by the isolates, even if they belong to different genotypic ranks. As reported elsewhere [19], physiological and biochemical criteria used for LAB strain identification are often ambiguous because most of the bacteria have very similar nutritional requirements and grow under similar environmental conditions. Therefore, a clear identification to species level by simple phenotypic tests may be troublesome and inaccurate. Molecular methods used for discrimination of LAB strains to genus and species level are more efficient than phenotypic approaches.

A preliminary study on LAB associated with pollens having remarkable antimicrobial activity is reported here. The uses of these strains for biocontrol or bio-preservation purposes should be evaluated. Phenotypic traits do not reveal the real taxonomic position of some isolates; therefore, exploitation of other molecular methods for exact identification of these bacteria is of great scientific and practical interest. In addition, the search for new LAB exhibiting a wider spectrum of antimicrobial activities from pollen grains that can be used in human health, agriculture and the food industry is of great importance. Furthermore, studies on other biological and biotechnological criteria of these isolates as well as their safety aspects are necessary. Finally, it seems that pollens and possibly other beehive products are predominant sources for isolation of LAB with potent applications.

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Microbiological quality control of marketed pollen

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ABSTRACT

Microbiological quality control of 13 pollen samples purchased from local markets and 2 other samples provided by apiarists was investigated. TAMC ranged from 3.00 to 5.48 Log CFU/g. TMYC was between 2.3 and 6.99 Log CFU/g. Staphylococcus aureus was recovered with high density in 14 samples (up to 8.32 Log CFU/g) and Enterobactericeae count ranges from 4.18 to 8.018 Log CFU/g. Moreover, Salmonella spp. and Listeria spp. were detected in seven and ten pollen samples respectively. Potent toxinogenic molds isolated from pollen such as Aspergillus flavus, A. niger, A. alliaceus, Penicillium sp., Alternaria alternata, Alternaria sp., Monila sitophilia, Rhisomucor pusillus and Mucor hiemalis were characterized by conventional methods. In addition, analyzed pollen samples contain pathogenic members of Enterobacteriaceae (Salmonella sp. Shigella sp., Proteus mirabilis, Citrobacter diversus, Klebsiella sp., Escherichia coli, Providencia sp. and Enterobacter cloacae) as revealed by biochemical identification tests. Interestingly, this type of marketed pollen has poor microbiological aspect, unacceptable, and a hazardous food material.

Keywords: Pollen, quality control, mycotoxigenic fungi, enterobacteria, *Salmonella*, *Listeria*.

INTRODUCTION

For centuries, plant products have been used for a variety of functions. Trading of plants and plant products used for medicinal purposes were expanded in various societies **[1, 2]**. This situation probably stimulated by the fact that people believe that the consumption of natural products is healthier and safe than conventional medicine. As a plant product, pollen collected either by man or by honey bees, is used for nutritional and medicinal purposes. Because of its richness by proteins, amino-acids, carbohydrates, minerals, as well as vitamins, it was used as a dietary and a fortifying food. During the last few years, numerous pollen formulas are developed and marketed worldwide. Furthermore, researches that revealed the promising biological activities (therapeutic effects) of pollen extracts in recent years enhanced greatly the importance of pollen consumption in the society. In Algeria, as well as in other countries, pollen is used by people for various reasons, principally, as a weight raising food, general health wellbeing, as a fortifying agent and prostate hypertrophy treatment. In practice, apiarists collect pollen using pollen traps. These later are non sterile and may contain various and different microbial populations originated during either manufacturing, transport, storage or handling. In addition, from flowers to beehives, pollen is exposed to microbial contamination by dust, air, insects, animals, and man. Most bee combs in Algeria, are not far away from industrial zones and metropolitan activities. Therefore, during their foraging, bees collect other man manufactured products, such as patisseries, sweet cakes; which may be contaminated by human or animal wastes. From another aspect, apiarists give, usually, for their bees, home made syrup basically prepared from sugar, milk and water. These latter ingredients are sources for microbial contamination and suitable growth medium for bacteria and fungi. Bee-Pollen, after collection, will be dried, purified, packed, stored and marketed to the consumers without an effective

hygienic and sanitary control. During each step of this important manufacturing chain, pollen continues to load and kept the original microbial flora as the first step, drying (40 °C max), is insufficient to removing and/or reducing microbial populations. Pollen is a suitable carrier for Gram positive and Gram negative bacteria, fungi and yeasts. Fungi and some bacterial species are spore formers. Also, growth and aflatoxin production, carcinogenic secondary mycotoxins, have been reported by Pitta and Markaki **[3]**. In addirion, our preliminary study **[4, 5]** on pollen microbial flora revealed the existence of elevated numbers of molds, coliforms, *Staphylococcus aureus* as well as aerobic and anaerobic microorganisms **[6]**. Some of these bacteria like *Bacillus cereus* and *Clostridium perfringens* are recognized as potential pathogenic organisms and have been incriminated in food poisoning **[7, 8]**. Pollen formation (flowering) and harvesting occur during warmth and humid seasons. Such environmental conditions favour microbial contamination and proliferation. Pollen can be classed as important vehicle for various micro-organisms implicating possible health problems for consumers and shelf life problems. Foodborne diseases are perhaps the most wide-spread health problem in the contemporary world and an important cause of reduced economic productivity **[9]**. In most of the cases of foodborne illness, the pathogenic effect occurs in the alimentary tract giving rise to symptoms of diarrhoea and vomiting. Since it is a natural product, all pollen constituent can be degraded by bacteria and fungi. Unscientific methods of harvesting, inappropriate drying and purification, unsuitable packing, storage and transportation, inadequate hygiene of producers and congenital climatic conditions render the raw material prone to infestations and exposed it to many microbial contaminants. Raw plant materials are most often degraded by microorganisms before harvesting, during handling and after prolonged storage **[10, 11]**. The presence of sufficient numbers of microorganisms can be harmful to consumers. As a result of fungal contamination, the risk of mycotoxin production, especially aflatoxins, should be taken into consideration in the manufacturing process because of the proven mutagenic, carcinogenic, teratogenic, neurotoxic, nephrotoxic, immunosuppressive activities **[12, 13, 14, 15]**. From a legal point of view, only limited countries worldwide have a legislation code for pollen manufacturing **[16]**. Thus, mush more studies for understanding the microbial load and critical quality control levels for pollen and pollen products are necessary. In this study, 15 samples of marketed pollen were analysed for their microbial content. Isolates of microbial contamination indicators were subjected for identification and antibiotic susceptibility testing.

MATERIALS AND METHODS

Sampling

A total of 15 samples of pollen were collected from local public markets. 13 samples were packed in glass or plastic containers without vacuum and 2 hand-collected other samples were freshly obtained from bee-keepers. All the samples (150 g each) were transported to the laboratory and stored at 4°C, until testing. They were analysed within 24 h of sampling. Prior to analysis, 25 g of each sample was homogenized for 10 min with 225 ml of 0.1% sterile peptone water (10^{-1} dilution). Serial dilutions were performed as required. The pH of the food samples was measured using a digital pH meter (Hanna 8417, Italy) in a 1:10 (w/v) mixture of the homogenate in sterile distilled water. Moisture of each sample was measured after drying three subsamples (1 g each) at 95°C until the obtention of constant weight.

Total aerobic plat counts

The pollen samples of which dilution has been prepared were plated on plate count agar (PCA). Plates were incubated at 30°C for 72 hours aerobically.

Enumeration and identification of molds

From each dilution in peptone water, 0.1 mL was spread onto potatoes Dextrose Agar (PDA). The plates were incubated at 25°C for 5 days. Each distinct mould colony was observed microscopically for morphological characterization and identification **[14, 15]**.

Enumeration of Enterobacteriaceae

Enterobacteria were counted by transferring a 100 µL aliquot of the appropriate dilution into Petri plates and poured with Mac Conkey agar and Brilliant Green Lactose Bile broth (BGLB). Plates were incubated at 35°C and typical colonies were counted after 24 h of incubation and subjected for biochemical identification.

Isolation of Salmonella spp.

Salmonella spp. was detected in 4 steps. Pre-enrichment (25 g of pollen in 225 mL in buffered peptone water) at 37°C for 16-20 h, was followed by enrichment in Rappaport-Vassiliadis (RV) broth incubated at 42°C for 24 h. The isolation was done on xylose lysine desoxycholate (XLD) agar at 37°C for 24 h. The colonies on the XLD agar plate were transferred to a triple sugar iron agar slant (TSI, Becton, Dickinson and Company) and incubated at 35°C for 24 h. The colonies on the TSI agar slant were chosen for identification based on morphological and biochemical tests.

Isolation and identification of Staphylococcus aureus

Enrichment of the bacteria was done by adding one gram (1g) of the sample into peptone water and incubated for 18 h at 37°C. Isolation of the *Staphylococcus aureus* was achieved by streaking the pre-enriched culture from the peptone water on to a selective differential agar plate of Baird-Parker Agar (BPA) which was freshly prepared following manufacturer's instructions. The plates were then incubated at 37°C for 24 h under aerobic conditions. Suspected colonies of being *S. aureus* (circular, smooth, convex, moist, and gray to jet-black, frequently associated with an outer clear zone) were subjected to biochemical tests.

***Detection of Listeria* spp.**

Twenty-five grams of pollen was homogenized in 225 mL of Listeria-Enrichment Broth (LEB) for 48 h at 37°C. Loopful of culture was streaked on to Listeria-Selective Agar (LSA). Characteristic positive colonies were picked up and subcultured in Brain Heart Infusion Broth (BHI) at 37°C for 24 h and conserved in the same broth containing glycerol (15%, v/v) at 5°C for further biochemical confirmation.

RESULTS

Moisture and pH

Fifteenth pollen samples purchased from local Algerian markets were subjected for microbiological analysis. Moisture and acidity (pH) of the samples were also measured (Table 1), pH values varied from 4.55 (from Mila sample) to 6.29 (from Egypt sample and Biskra sample). Statistical analyses indicate that there was no significant difference between pH values of analysed pollen samples ($P > 0.05$). In addition, moisture content of pollen ranged from 18.11% to 36.29% (samples from Egypt and Constantine respectively). Raw pollen obtained from Biskra and Constantine has the highest moisture content (3.036 and 36.29% respectively, Table 1). A two-way ANOVA test revealed that there was a significant difference ($P < 0.05$) between relative humidity of pollen samples PS-Alg1/PS-Egy, PS-Alg2/PS-Cons, PS- Chi1/PS-Egy, PS-Alg3/PS-Cons, PS-Alg4/PS-Cons, PS-Bli/PS-Cons, PS-Msi/PS-Cons, PS-Bis/PS-Egy, PS-BBA/PS-Cons, PS-Syr/PS-Egy, and PS-Egy/PS-Chi2. Furthermore, extremely significant difference between moisture of pollen from Egypt and Constantine ($P < 0.001$) was observed.

Table 1: Moisture, pH and microbial count for 15 pollen samples. Results are expressed as Log CFU/g of wet pollen and presence (+) or absence (-) for *Salmonella* and *Listeria*

Pollen samples	TAMC[a]	TMYC[b]	*Staphylococcus aureus*	Enterobacteria	*Salmonella* spp.	*Listeria* spp.	pH	Moisture (%)
PS-Alg1	3.30	2.770	2.477	7.18	+	+	4.72	28.42
PS-Alg2	3.30	2.690	2.903	ND	-	-	5.11	25.79
PS-Chi1	3.60	3.450	ND	ND	-	+	6.03	27.92
PS-Sét	3.95	3.230	3.301	8.016	+	-	5.86	26.85
PS-Alg3	3.85	2.300	2.903	5.38	+	+	4.97	25.43
PS-Alg4	3.60	2.300	2.903	6.96	+	-	5.33	25.48
PS-Bli	5.48	2.850	2.602	5.72	+	+	5.18	26.58
PS-Mil	4.56	4.090	3.204	ND	-	+	4.55	27.57
PS-Msi	3.70	2.480	2.301	ND	-	+	5.30	25.14
PS-Bis	5.49	6.920	7.890	4.18	+	+	6.30	30.36
PS-BBA	3.30	3.080	2.477	ND	-	+	5.19	26.18
PS-Syr	3.57	3.000	2.477	4.32	-	-	5.51	28.36
PS-Egy	ND[c]	6.990	8.320	7.67	+	+	6.29	18.11
PS-Chi2	ND	4.480	2.778	4.20	-	-	6.14	27.90
PS-Cons	ND	4.210	6.420	6.41	-	+	5.38	36.29
Safety criteria (Campos *et al.* 2008)	< 5 Log CFU/g	< 4.7 Log CFU/g	Absent/1 g	< 2 Log CFU/g	Absent/10 g	/	/	/

[a] TAMC. Total aerobic mesophylic count; [b] TMYC. Total molds and yeasts count; [c] ND. not detected; +. presence; -. absence. PS: pollen sample, PS-Alg1: Algiers1, PS-Alg2 : Algiers2, PS-Alg3 : Algiers3, PS-Alg4 : Algiers4, PS-Chi1 : China1, PS-Chi2 : China2, PS-Sét : Sétif, PS-Bli: Blida, PS-Mil: Mila, PS-Msi: M'sila, PS-Bis: Biskra, PS-BBA: Bordj Bou-Arreridj, PS-Syr: Syria, PS-Egy: Egypt, PS-Cons: Constantine.

Total Microbial Load

Total microbial count indicates that all pollen samples with the exception of pollen from Egypt, second sample from China and sample from Constantine (CFU=0), have less or more microbial load (Log CFU/g = 3.00 for pollen from Syria to Log CFU/g = 5.48 for pollen from Blida, Table 1). Furthermore, height fungal loads was observed for the pollen sample from Biskra and from Egypt sample (Log UFC/g = 6.92 and 6.99 respectively, Table 1), an intermediate fungal content (Log UFC/g=2.3 to 4.48) for the other samples. Statistical analysis (Two-way ANOVA

test) indicates that there were no significant differences between values total microbial count as well as fungal count for all pollen samples ($P > 0.05$).

Table 2: Fungal species isolated from pollen samples

Molds	Samples														
	PS-Alg1	PS-Alg2	PS-Chi1	PS-Sét	PS-Alg3	PS-Alg4	PS-Bli	PS-Mil	PS-Msi	PS-Bis	PS-BBA	PS-Syr	PS-Egy	PS-Chi2	PS-Cons
Aspergillus flavus		+							+		+	+			
A. niger	+		+								+	+			
A. alliaceus			+	+										+	+
Penicillium sp. 01		+				+				+					
Penicillium sp. 02		+			+								+		
Penicillium sp. 03				+											
Penicillium sp. 04				+											
Penicillium sp. 05					+										
Penicillium sp. 06				+											
Alternaria alternata				+				+						+	
Alternaria sp. 01				+			+								
Alternaria sp. 02				+			+								+
Monila sitophilia							+								
Cladosporium werneckii				+								+			
Drechslera tritici-repentis				+											
Verticillium albo-atrum	+				+				+				+	+	
Rhisomucor pusillus	+				+										
Mucor hiemalis					+										
Sepedonium chrysospermum				+				+	+						
Phialophora verrucosa	+	+				+	+	+						+	
Monascus ruber								+							
Geotrichum candidum							+								+

Pathogen Content

Considering now *Staphylococcus aureus*, the absence of these bacteria only in one pollen sample imported from China. The bacterial density of the other samples varied from 2.30 to 8.32 Log CFU/g. The highest was for pollen imported from Egypt (8.32 Log CFU/g) followed by 7.89 Log CFU/g for pollen from Biskra (Table 1). The average of *Staphylococcus aureus* density for the other samples ranged from 2 to 4 Log CFU/g (Table 1). A significant statistical difference ($P < 0.05$) exists between Log CFU of one sample imported from China and pollen sample from Biskra. However, a very significant difference ($P < 0.01$) was also observed between values the same china's pollen sample and that for the sample imported from Egypt. No statistical difference was observed for values of enterobacteria count ($P > 0.05$).

The average of enterobacteria count ranged from 4.18 to 8.016 Log CFU/g for ten samples. In opposite, counts of enterobacteria for the other five samples were negative. It seems that samples from Sétif, Egypt, Constantine, Blida and Algiers were very contaminated (5.38 to 8.016 Log CFU/g). As another indicator of fecal contamination, *Salmonella* was recovered from seven samples (Table 1). Elevated bacterial density was observed. Only one sample of imported pollen (from Egypt) contains *Salmonella* spp., the other analyzed samples which contain these bacteria were locally produced. Also, *Listeria* was detected in 10 samples, from which 2 were imported, one from China and another from Egypt (Table 1).

Microbial identification

Twenty-two fungal isolates were assigned to species level based on morphological characteristics. They were characterized to 16 different species (*Aspergillus flavus, A. niger, A. alliaceus, Penicillium* sp., *Alternaria alternate, Alternaria* sp., *Monila sitophilia, Cladosporium werneckii, Drechslera tritici-repentis, Verticillium albo-atrum, Rhisomucor pusillus, Mucor hiemalis, Sepedonium chrysospermum, Phialophora verrucosa, Monascus ruber* and *Geotrichum candidum*) (Table 2). Furthermore, From Mac Conkey agar plats, several and morphological different colonies derived from the culture of each pollen sample were subjected for biochemical characterization. Table 3 shows results of the identified isolates belonging to enterobacteria.

Table.3: Bacterial isolats belonging to enterobacteria recovered from pollen samples

Number of isolats	Pathogen identity	Pollen samples in which pathogen was recovered
12	*Salmonella* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét
25	*Shigella* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1
30	*Proteus mirabilis*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
28	*Citrobacter diversus*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
14	*Klebsiella* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
26	*Escherichia coli*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2
19	*Providencia* sp.	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Chi2, PS-Mil, PS-Msi
29	*Enterobacter cloacae*	PS-Alg1, PS-Bli, PS-Alg3, PS-Alg4, PS-Sét, PS-Cons, PS-Mil, PS-Msi, PS-BBA, PS-Syr, PS-Chi2, PS-Chi1, PS-Alg2

DISCUSSION

Plant products have been identified and confirmed as a significant source of pathogens and chemical contaminants that pose a potential threat to human health worldwide. There has been a growing interest in developing and applying microbiological criteria to the sanitary control of beehive products especially pollen. Ideally such standards should be based on bacterial counts associated with disease. Instead, a standard related to sanitation practices might specify the permissible number of microorganisms in an acceptable food product derived from CFU counts obtained in a large-scale study on representative pollen samples. Usefulness of these study data is limited since the study was designed to survey microbial counts in only fewer samples **[17]**.

In the present study, 15 pollen samples were analysed. Their acidity, moisture, microbial load and pathogen content were determined. Enormous total aerobic mesophylic count (TAMC) was recorded for 12 samples (>3 Log CFU/g). In three samples total aerobic mesophylic microorganisms was nil even their pH values were near neutrality and they have low moisture. It seems that microorganisms in these samples were in dormant stat and needs longer period than that of incubation time for adaptation to the laboratory chemical and physical growth conditions. Furthermore, total molds and yeast count (TMYC) was also important. All pollen samples have remarkable fungal density. Comparing our results with that recommended by Campos *et al*. **[16]** (TAMC not exceed than 5 Log CFU/g and a TMYC less than 4.7 Log CFU/g), three pollen samples (PS-Bli, PS-Bis, and PS-Egy) were out of safety recommendations. In addition, according to the same safety criteria (Absent of *Staphylococcus*/1 g and enterobacteria should be less than 2 Log CFU/g); all pollen samples (except that imported from China, PS-Chi1) were of pour microbiological quality. Ten samples contain more than 2 Log CFU of enterobacteria/g, and 14 samples contain more than 8 Log CFU of staphylococci per gram of pollen. More importantly, the detection of *Salmonella* in seven pollen samples is a direct evidence for a fecal contamination, and renders the food material a potentially product and indicates poor food handling practices.

In fact, the test for *Enterobacteriaceae* has replaced the tests for coliforms that traditionally have been used as indicators of hygiene and contamination after processing. The major problems with the coliform tests are the variability in definition of the term coliforms (they are defined usually by the method used for their detection) and the fact that only lactose fermenting organisms are detected. In comparison the family *Enterobacteriaceae* is well defined taxonomically and methods for their enumeration are based on common properties. Furthermore, the methods also detect important non-lactose fermenting organisms such as *salmonellas* **[18]**. Also, *Listeria* spp. other than *L. monocytogenes* are rarely implicated in illness. They are indicators for the likely presence of *L. monocytogenes*. Furthermore, this pathogen is widely distributed in the environment and is able to multiply slowly at 4°C. The shelf life of foods varies enormously, and the presence of *L. monocytogenes* at any level may be of significance due to its potential for growth during storage. The use of an enrichment procedure, in addition to enumeration, should therefore be considered to ensure that the organism is absent from the product.

From another point of view, the slightly acidic aspect of the analyzed pollen samples favors the development of fungi and mycotoxin production. *Aspergillus, Penicillium, Alternaria, Mucor* and others were recovered from pollen. They are involved in various diseases such as allergic illness, mycotoxicosis, and aflatoxicosis **[19]**. In addition, the recovery of potent pathogenic bacteria belonging to the family *Enterobacteriaceae* in another proof of unsatisfactory and unacceptable pollen set for human consumption in local markets. A greatly intension should be taken developing legislation considering pollen and pollen extracts as well as standards for microbial quality control

of this type of products. More importantly, expanded studies about pollen processing and preservation to reduce microbial load pathogen elimination are necessary.

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مـلـخـص

في هذا البحث، تم التحقق من الجودة الميكروبيولوجية لعينات من طلع النحل. تراوح تعداد الفلورا الكلية الهوائية بين 3 و 5 لغ وم م / غ، أما الفطريات فقدرت بين 2.3 و حوالي 7 لغ وم م /غ. ومع ذلك، لوحظت كثافة عالية للمكورات العنقودية الذهبية (تصل إلى 8.32 لغ وم م / غ). فيحين فتعداد بكتيريا القولون تأرجح من 3 إلى أكثر من 8 لغ وم م / غ. بالإضافة إلى ذلك، تم الكشف عن الأمعائيات الممرضة و عزلت الفطريات المنتجة للسموم. كما انتشلت 567 عزلة من بكتيريا حمض اللبن. اعتمادا علي النشاطية العدائية تم تحييد 54 بكتريا حمض اللبن من بين 216 عزلة ذات نشاطية حيوية. علي أساس الصفات المظهرية، حددت سبعة مجموعات مع عضوين آخرين على مستوى التشابه 79 ٪. كما بين التسلسل الجزئي لمورثات الرنا الريبوزي 16S، أن عشر سلالات من البكتيريا اللبنية تنتمي إلى سبعة أنواع : *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus ingluviei*, *Pediococcus pentosaceus*, *Lactobacillus acidipiscis* , *Weissella cibaria* (*Lactobacillus*) بعد ذلك ، عشر سلالات من العصيات اللبنية شخصت علي أساس الميزات المظهرية و الوراثية اختيرت لمزيد من تقييم خصائص التعزيز الحيوي. ففي درجة الحموضة 2، لم يتمكن من انتشال سلالتين فقط (*Lactobacillus* sp. BH1398 وBH1480) بعد ثلاث ساعات من التجربة. ثبت أن الأنواع *Lb. plantarum*, *fermentum*, *Lb.acidophyllus*, *Lb. pentosus* هي الأكثر مقاومة لهذه الظروف القاسية. بالإضافة إلى ذلك، في درجة الحموضة 3 يمكن لجميع السلالات النمو. و يبدو أن، 0.3٪ و 0.5٪ من الأملاح الصفراوية لا تؤثر بشكل كبير على بقاء معظم السلالات ، باستثناء *Lactobacillus* sp.BH1398 . أبدت كل السلالات مقاومة للكوليستين، الكليندامايسين، الكلورامفينيكول، وسيبروفلوكساسين، لكن معظم السلالات حساسة للمضادات الحيوية، أوكساسيلين، أوكسيتيتراسيكلين، وأموكسيسيلين. و أخيرا تم التحقق من أمكانية *Lactobacillus fermentum* BH1509 بشأن تأثيرها الكابح ضد سلالتين من سلالات *Helicobacter pylori*. قيس الفعل القاتل للمحلول الطافي لمستنبت البكتيريا اللبنية. و تنتج السلالة BH1509 مبيدا ميكروبيا (بكتيريوسين) منخفض الوزن الجزيئي، سمي BAC-F15. بعد تنقية جزئية بالترسيب بسلفات الأمونيوم و كروماتوغرافيا Sephadex G-50، قدرالوزن الجزيئي التقريبي لـ BAC-F15 بعد الهجرة الكهربائية بين 4100 و 6500 دالتن. يبدي هذا البكتيريوسين نشاطية تثبيطية واسعة ضد بكتيريا فساد الأغذية و الممرضة، بما في ذلك البكتيريا إيجابية الجرام مثل *Micrococcus luteus*, *Staphylococcus aureus*, *Listeria innocua* وسالبة الجرام، *typhimurium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Helicobacter pylori* ، *Campylobacter jejuni* . إضافة لذلك، لم يتأثر نشاط السائل الطافي المضاد للبكتيريا بالكاتالاز أو الليباز، لكن ألغي من قبل الإنزيمات المحللة للبروتين مثل بروتيناز K، التربسين و بروناز E. BAC-F15 هو بروتين مقاوم للحرارة (10 دقيقة في 100 درجة مئوية) و أظهر نشاطا مثبطا على نطاق واسع من درجة الحموضة (4.0-8.0). أنه يبدي فعلا قاتلا للجراثيم كما يتضح من نمط تأثيره على سلالات *Helicobacter pylori*. إن المبيد الميكروبي والبكتيريا المنتجة له، *Lactobacillus fermentum* BHB1509، قد تكون مضادات ميكروبية مفيدة للسيطرة على الالتهابات المعوية التي تحدثها *Helicobacter pylori*

ABSTRACT

In this research, microbiological quality control of pollen samples was investigated. TAMC ranged from 3.00 to 5.80 Log CFU/g. TMYC was between 2.3 and about 7 Log CFU/g. However, *Staphylococcus aureus* was recovered with high density (up to 8.32 Log CFU/g). *Enterobactericeae* count ranges from 3.00 to more than 8 Log CFU/g. In addition, samples contain pathogenic members of *Enterobacteriaceae* and potent toxinogenic molds Furthermore, 567 isolates of lactic acid bacteria were recovered from raw bee pollen grains. Based on antagonistic activity, neutralized supernatants of 54 lactic acid bacteria (LAB) cultures from 216 active isolates inhibited the growth of indicator bacteria. They were phenotypically characterized and seven clusters with other two members were defined at the 79% similarity level. Partial sequencing of the 16S rRNA gene of representative isolates, 10 strains were assigned to seven species: *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus ingluviei*, *Pediococcus pentosaceus*, *Lactobacillus acidipiscis* and *Weissella cibaria*. After that, 10 *Lactobacillus* isolates identified, by phenotypic and genotypic methods, were selected for further evaluation of their probiotic properties. At pH 2, only tow strains (*Lactobacillus* sp. BH1398 and BH1480) that could not recovered after three hours. In addition, at pH 3 all strains could grow and resist the acidic conditions. It seems that, 0.3% and 0.5% of bile salts does not affect greatly the survival of most strains, excluding *Lactobacillus* sp. BH1398. In contrast, in the presence of 1.0% bile salts, survival of five strains was decreased by more than 50 %. In addition, all strains were resistant to colistine, clindamycine, chloramphenicol, and ciprofloxacine, but most of the strains were susceptible to peniciline, oxacillin, Oxytetracyclin, and amoxicillin. Finally, *Lactobacillus fermentum* BH1509 was investigated for its inhibitory effect against two *Helicobacter pylori* strains. The bactericidal activity of spent culture supernatants (SCS) was measured. The strain BH1509 produced a small bacteriocin, designated Bac-F15. After partial purification by ammonium sulfate precipitation and Sephadex G-50 chromatography, the approximate molecular weight of Bac-F15 was estimated by SDS-PAGE between 4100 and 6500 Da. It displayed a wide inhibitory spectrum against food-spoiling bacteria and food-borne pathogens. The antibacterial activity of cell-free culture supernatant fluid was not affected by catalase or lipase but was abolished by the proteolytic enzymes proteinase K, trypsin and pronase E. Bac-F15 was heat stable (10 min at 100°C) and showed inhibitory activity over a wide pH range (from 4.0 to 8.0). It has a bactericidal action as evidenced by its action upon *Helicobacter pylori* strains. The bacteriocin and its producer, *Lactobacillus fermentum* BHB1509, may be useful antimicrobial materials to control gastrointestinal infections caused by *Helicobacter pylori*.