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Population Dynamics of Lactic Acid Bacteria during Laboratory Fermentation of Honeybee-Collected Pollen

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ABSTRACT

A preliminary study on fermentations of poly-floral and date palm pollens by their native and complex LAB flora in selective medium indicates the succession of different LAB major genera (enterococci, lactobacilli, pediococci/leuconostocs and streptococci/lactococci) with shared growth pattern and height bacterial density (6.28-7.5, 6.86-7.54, 6.20-7.20, and 5.60-6.80 Log_{10}CFU/mL respectively). Synergistic effect between bacterial groups was observed and antagonism was another phenomenon (at least by lowering pH, 3.46-4.0) influencing the dynamics of LAB population during laboratory scale pollen fermentation.

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 [1] 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 [2, 3]. Nilsson [4] found that the predominant LAB during silage fermentation were streptococci and lactobacilli, with *Lactobacillus plantarum* the species most frequently recovered. Other studies [5, 6] 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* [7]. 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.

MATERIALS AND METHODS

Enrichment and enumeration of LAB.

Two pollen samples were diluted (20%, w/v) in Elliker broth pH 6.5 [8]. 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. Form each enrichement culture, serial dilutions were prepared as needed in peptone water (0.1 % peptone and 0.1 % of Tween 80), and from the appropriate dilution, aliquots (100 µL) were cultured on the following media: M17 agar (Fluka) incubated for 72 hr at 30°C for lactococci and streptococci, LBS agar [9] after anaerobic incubation (BBL GasPack System) at 30°C for 72 hr for lactobacilli, Glucose Yeast Extract Agar [10] 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.

RESULTS

Figure 1 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. 1A) 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 reaches 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 decline to reaches 6.20 Log CFU/mL at the end of the fermentation (Fig. 1A). All LAB groups studied 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 decreases to a minimum level of 3.46 (Fig. 1A). 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. 1B). A first phase, characterized by the increase of LAB density, except for lactobacilli, which were appears only after 18 h, followed in general by a remarkable increase of bacterial density, and then the growth enter a stationary phase. Enterococcus reaches 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. 1B). The population size of the enterococci decrease 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 appears to reaches 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. 1B).

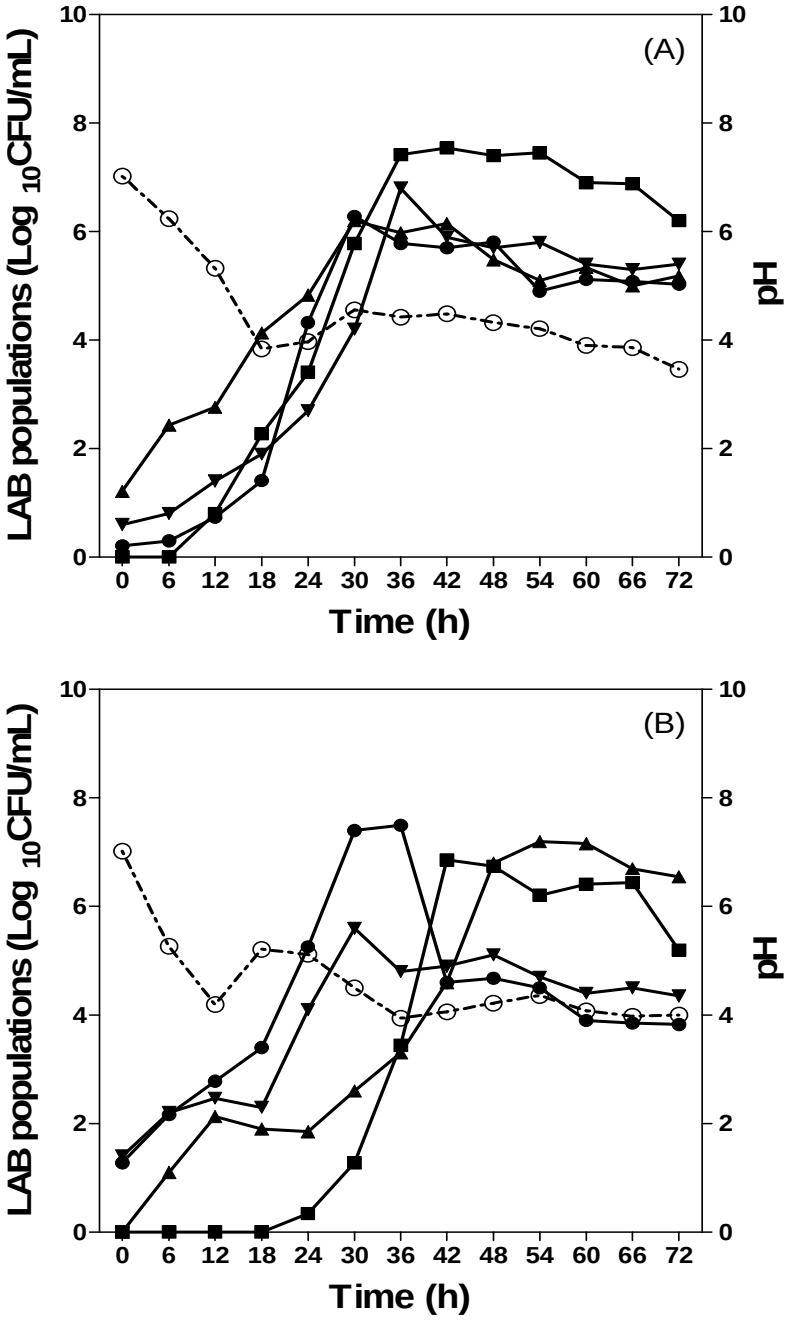

Figure |1
Lactic acid bacteria (LAB) populations during fermentation of poly-floral pollen (A) and monofloral (B) (date palm pollen). Microbial succession (Log CFU/mL) of enterococci (-●-), lactobacilli (-■-), pediococci and leuconostocs (-▲-), streptococci and lactococci (-▼-), Culture pH (-○-) variation during 72 hour of incubation

DISCUSSION

Fermentation of pollen grains was addressed only by Pain and Maugenet [11]. 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.). 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 **[12]**. Pollen microbial flora 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. 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 exhibits 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. 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 completion for nutriments as another mechanism involved natural in poly-microbial systems. This antimicrobial of pollen associated LAB was in vitro demonstrated previously [13]. 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.

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