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Microbiology of Ripening Honey

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Two main groups of bacteria, classified as *Gluconobacter* and *Lactobacillus*, are present in ripening honey. A third bacterial group, classified as *Zymomonas*, and several types of yeast are occasionally isolated. Both in natural honey and in synthetic syrup the bacterial population decreases in the course of the ripening process. *Lactobacillus* and *Gluconobacter* disappear after minimum moisture (about 18%) is reached, but the former does so sooner than the latter. The presence of these bacteria in different parts of the bee has been also investigated.

Nectar is converted into honey through a maturation process. The most prominent feature of this process is a considerable water loss (40 to 70% of nectar initial weight) that takes place in two stages: an initial evaporation carried out by the bee, which brings down water content to 40 to 50%, and the final evaporation that takes place in the honeycomb, which yields a product with 15 to 18% water. Besides dehydration, a number of chemical transformations have been detected (2). A considerable microbial population exists in the initial stages of maturation and may be involved in some of these transformations (16, 19). We report here on microorganisms found in ripening honey and their possible relationship with other microbial groups in the beehive.

MATERIAL AND METHODS

Honey samples. Honey at different ripening stages was collected from honeycombs in situ with the aid of a wide-tipped pipette, and dilutions in 10% (wt/vol) glucose were made for microbial counting and isolations. The apiary was located near Madrid, in an area with a flora of *Rosmarinus officinalis* and other *Labiatae* (*Lavandula* spp. and *Thymus* spp).

Bees. Bees from the same apiary were dissected, and the appropiate anatomical parts were homogenized in a blender with sterile 10% sucrose solution for microbial counting and isolation.

Media and culture conditions. Yeast-glucose broth (0.6% [wt/vol] yeast extract, 5% [wt/vol] glucose), with or without 1.5% (wt/vol) agar, was used for the growth of bacteria unless stated otherwise. Wickerham agar (23) was used for yeast. Temperature of incubation was 30 C for bacteria and 28 C for yeast.

Lactobacilli were maintained in stabs of yeast-glucose broth with 0.5% (wt/vol) agar and 1% (wt/vol) $CaCO_3$. Fresh isolations of *Gluconobacter* were maintained in yeast-glucose broth, with transfers every 5 days, for periods under 1 month. Freeze drying was used for longer conservation periods.

Counting procedures. *Gluconobacter* was counted as previously described (19), except that 0.3% instead of 0.6% yeast extract was used to stimulate acid production by *Gluconobacter* and to limit the growth of lactobacilli.

Lactobacilli were counted by plating in MRS agar (11) with 10% (wt/vol) glucose instead of 2% (wt/vol). In this medium growth of *Gluconobacter* is very poor.

Zymomonas and yeast were counted in Wickerham agar (23), in which they can be distinguished from each other.

Identification of bacteria. Procedures recommended by the Society of American Bacteriologists (21) were essentially followed, except that 0.6% (wt/vol) yeast extract medium was used instead of nutrient broth.

The following methods were used as previously published: NH_3 production from arginine (14); benzidine test (5); gas production (6); vitamin requirements (18); and fermentation of sugars (11, 21).

Ethanol tolerance was observed in yeast-glucose broth.

Identification of yeast. Species indentification was done according to Lodder (9).

Simulation of the ripening process. *Gluconobacter* and lactobacilli, isolated from honey and grown in 0.6% (wt/vol) yeast extract with 30% (wt/vol) glucose, were added to a syrup of the following composition: glucose (10 g), fructose (10 g), yeast extract (0.06 g), KCl (48 mg), and water (30 g). The mixture was placed in a special, 250-ml, wide-mouthed Erlenmeyer flask and shaken in a New Brunswick orbital shaker at 30 C until 45% moisture was reached (7 to 8 h), and then evaporation was allowed to proceed without shaking down to 16 to 18% moisture. Further dehydration was prevented by sealing the flask with Parafilm.

Chemical methods. Acidity and moisture of honey and sugar syrup were determined by methods of the Association of Official Agricultural Chemists (1). Sugars, ethanol, mannitol, and acidity of culture media were analyzed according to Neish (13). Mannitol determination was carried out only in samples practically devoid of fructose. D- and L-lactic acids were quantitated with the aid of D-lactate dehydrogenase (Boehringer, no. 15002) and L-lactic dehydro-

genase (Serva, no. 27403) by the method of Cato and Moore (20). A quantitative Van Iterson-Kluyver fermentometer (A. J. Kluyver, Ph.D. thesis, Delft Univ. of Technology, Delft, The Netherlands, 1914) was used for CO_2 determinations.

RESULTS

Types of microorganisms in ripening honey. Two main groups of microorganisms are found in ripening honey: *Gluconobacter* and *Lactobacillus*. Also present are a group of ethanol-producing bacteria and different types of yeast.

The first group, *Gluconobacter*, has been previously described by us (19).

We classify the second group as *Lactobacillus viridescens* on the basis of the following characteristics (17).

They are gram-positive rods (0.5 to 0.7 by 1.5 to 3 μm), occurring singly or forming pairs at an angle; nonmotile; and nonsporing. A few strains show a brick red pigment.

They are microaerophilic to aerobic; catalase and benzidine reactions are negative.

They are heterofermentative, and acid and gas are produced from fructose; acid can be detected from glucose, sucrose, maltose, mannitol, and raffinose. Pigmented strains also ferment xylose, arabinose, ribose, and trehalose. No acid or gas is produced from lactose, melibiose, melezitose, ramnose, sorbose, inulin, salicin, starch, sorbitol, adonitol, erythritol, dulcitol, and inositol. The principal products from fructose are mannitol, DL-lactic acid, acetic acid, and CO_2; the principal products from glucose are DL-lactic acid, acetic acid, and ethanol. The growth on glucose is slow and scanty, which could account for our failure to detect CO_2 production from this sugar.

All strains are negative in tests for production of indole, growth on litmus milk, reduction of nitrates, gelatin hydrolysis, production of NH_3 from arginine, and growth in 15% ethanol.

Optimum temperature for growth is 30 to 32 C; no growth occurs at 45 or 10 C, growth occurs at 15 C.

It should be pointed out that, in a preliminary study, these bacteria had been tentatively classified as *Brevibacterium* (16).

The ethanol-producing bacteria are easily detected when present in ripening honey because of their outstanding gas-producing ability. These bacteria have the following characteristics.

They are gram-negative, catalase-positive short rods (1.5 to 2 by 2 to 3 μm), usually in pairs.

One mole of glucose is fermented with the production of 1.8 mol of ethyl alcohol and 1.9 mol of CO_2. Sucrose and maltose are also fermented. Slight growth, but not gas, is produced from galactose, mannitol, raffinose, and melezitose. Slime is produced from sucrose.

Optimum temperature for growth is 25 to 32 C; no growth occurs at 15 or 45 C.

We classify these bacteria as *Zymomonas mobilis* (4).

Different types of yeast are isolated from ripening honey. We have found four types, numbered 1 to 4 in a decreasing frequency order. Their characteristics are summarized in Table 1. According to these characteristics, we classify the four types as follows: type 1, *Torulopsis magnoliae*; type 2, *Saccharomyces mellis*; type 3, *Torulopsis stellata*; and type 4, *Torulopsis apicola*.

Number of microorganisms. It has been consistently found that the number of *Gluconobacter* and *Lactobacillus* in cells half-filled with high-moisture honey, at the time of maximum flowering, is much higher than in cells ready to be sealed and filled with low-moisture honey. A significant correlation exists between moisture and log of population number, both for *Gluconobacter* and for *Lactobacillus* (Fig. 1).

Bacterial populations in nectar extracted from the bee's stomach are bigger than those found in the honeycomb: an average of 2×10^5 *Lactobacillus* and 10^5 *Gluconobacter* in stomach versus 3×10^3 found in honeycomb samples with the highest moisture (see Fig. 1).

Only a low proportion of ripening honey samples have *Zymomonas* or yeast: 2 out of 10 samples and 5 out of 10 samples, respectively. Up to 1,000 *Zymomonas*/g and 100 yeast/g have been found.

Distribution of honey microorganisms in

TABLE 1. *Characteristics of yeast isolated from ripening honey*[a]

Test	Type			
	1	2	3	4
Ascospores formation	−	+	−	−
Fermentation				
Sucrose	+	−	+	+
Raffinose	−	−	+	±
Assimilation				
Sucrose	+	−	+	+
Galactose	±	+	−	−
Raffinose	+	−	+	+
Mannitol	+	+	−	+
L-Sorbose	+	+	−	+
Nitrates	+	−	−	−
Growth on 60% glucose	+	+	±	+

[a] All strains share the following characteristics: fermentation, glucose +, galactose −, lactose −, melibiose −, trehalose −; assimilation, lactose −, maltose −, cellobiose −, melibiose −, melezitose −, L-rhamnose −, trehalose −, inulin −.

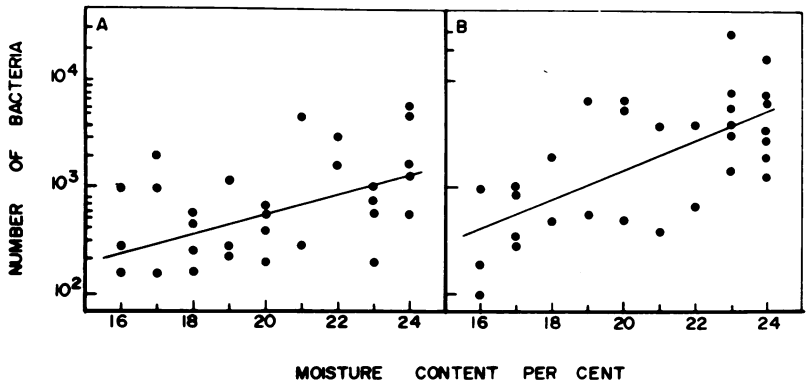

FIG. 1. *(A) Number of Gluconobacter found in samples of ripening honey plotted versus their moisture content. Slope of regression line log of bacterial number over moisture is $r = 0.10 \pm 0.03$ ($P < 0.01$). (B) As in (A) but for Lactobacillus; $r = 0.14 \pm 0.03$ ($P < 0.01$).*

the bee. All microorganisms associated with ripening honey are also found in the bee.

Gluconobacter, 10^3 to 10^6 per bee, are about equally distributed in the alimentary tract and the rest of the body. Within the alimentary tract, the mesointestine is the main reservoir. *Lactobacillus,* 10^4 to 10^7 per bee, are mainly located in the mesointestine, although significant numbers are found in the body surface.

No seasonal variation has been found in the number of *Gluconobacter,* whereas *Lactobacillus* are only present during the flowering period.

Both *Zymomonas* and yeast are ocassionally isolated from the bee, but they do not seem to be part of its constitutive microflora.

Simulated ripening. The evolution of *Gluconobacter* and *Lactobacillus* populations in a dehydrating syrup is plotted in Fig. 2. After a sharper initial drop, probably due to osmotic shock, the populations decrease slowly until they are not detectable (<1 bacteria/g). This takes place after reaching the final moisture level (17 to 18%) in both cases, but 2 days earlier in *Lactobacillus* (day 5) than in *Gluconobacter* (day 7).

Final total and lactone acidity, as well as pH, at different initial inocula in the syrup are presented in Table 2. Bacterial numbers of the same order as those found in ripening honey (10^4) do not affect the stated characteristics, but the higher inoculum does produce significant changes.

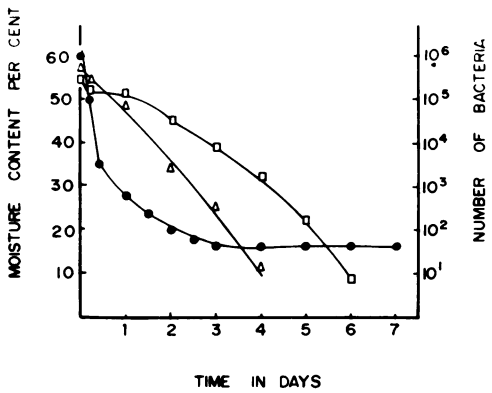

FIG. 2. *Evolution of Gluconobacter and Lactobacillus populations during simulated ripening of a synthetic syrup. Symbols: (●) Moisture content; (□) number of Gluconobacter; (△) number of Lactobacillus.*

TABLE 2. *Total acidity, lactone acidity, and pH after simulated ripening of a syrup initially inoculated with different numbers of Gluconobacter and Lactobacillus*

Inoculum (no. of each bacteria/g)	Acidity (meq/liter)		pH
	Total	Lactone	
0	3	0	5.9
10^4	3	0	5.9
5×10^6	20	5	3.9

DISCUSSION

There is little previous literature to which our results can be compared.

Bailey (2) found *Bacterium eurydice* (*Achromobacter eurydice* [3]) in the alimentary tract of the bee, as well as in larvae, pollen, and honey.

Pain and Maugenet (15) reported the presence in stored pollen of two groups of bacteria: a gram-negative one, classified as "close" to *Pseudomonas,* and a gram-positive one, classified as *Lactobacillus.* These bacteria contribute to pollen conservation, mainly through a lactic fermentation. The *Lactobacillus* was also present in just-deposited honey but disappeared as moisture decreased.

Vecchi and Zambonelli (22) isolated from the bee's mesointestine two groups of bacteria, classified as *Achromobacter eurydice* and *Brevibacterium*.

The characteristics reported by Vecchi and Zambonelli for *Achromobacter eurydice* are quite similar to those of our *Gluconobacter* (19), and, certainly, *Gluconobacter* is "close" to *Pseudomonas*.

The data concerning *Brevibacterium* (22) are also essentially similar to that reported here for *Lactobacillus*. Indeed, we had tentatively classified these bacteria as *Brevibacterium* (16).

It seems that a simple specific flora is present in the beehive, which is implicated in pollen conservation and possibly in honey ripening.

It is difficult to explain the low incidence of yeast in ripening honey. Before the final low water activity is achieved, proliferation of sugar-tolerant yeasts should be possible. It is well known that mature honey ferments readily if moisture is above 18% (8, 10). Mature honey has antibacterial activity but is not antifungic or antizymogenic (7). Thus, the low ocurrence and the low yeast populations in ripening honey could be explained in terms of an antizymogenic factor that disappears at maturity or as a competitive inhibition by the bacterial population.

Gluconobacter and *Lactobacillus* populations decrease as ripening proceeds; the number of bacteria decreases from stomach nectar to higher-moisture honey to low-moisture honey. It seems that *Lactobacillus* decreases more rapidly than *Gluconobacter* both in natural honey and in the synthetic syrup (Fig. 1 and 2).

It is difficult to establish the contribution of these bacteria to the ripening process. Our results indicate that a mixed population of *Gluconobacter* and *Lactobacillus* (5×10^6 bacteria/ml each) brings about in the synthetic syrup a final pH, as well as total and lactone acidities similar to those of natural honey. We have not detected such high populations in ripening honey, but we cannot claim to have estimated the maximum numbers reached. Although these maxima are not likely to be as high as the above figure, the contribution of the bacteria to the ripening process could still be significant.

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