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AEROBIC AND ANAEROBIC SPORE-FORMING BACTERIA IN SARDINIAN HONEY*

RIASSUNTO

I risultati della presente indagine hanno evidenziato che nei 52 campioni di miele esaminati, ad una microflora casuale ed ubiquitaria si associano altre forme a significato ben preciso. È il caso del *B. alvei* e del *B. larvae*, i quali possono avere effetti negativi sulla sanità delle popolazioni delle api; del *B. cereus*, spesso legato a tossinfezioni alimentari o ad alterazioni degli alimenti; del *Cl. beijerinckii*, responsabile di fermentazioni butirrica e acetica.

Per tali considerazioni è indispensabile che tutti i Paesi inseriscano nelle proprie legislazioni riguardanti il miele, anche i parametri microbiologici, al fine di garantire il consumatore sulla qualità e sanità del prodotto.

SUMMARY

Apart from an ubiquitous microflora, this investigation into 52 samples of honey revealed some undesirable spore-forming bacteria, *Bacillus alvei* and *B. larvae* which are bee pathogens. *Bacillus cereus* can cause spoilage and food poisoning.

It is, therefore, considered essential that every country includes microbiological standards in its Food Safety Regulations for honey, so that the consumer is guaranteed as to the wholesomeness as well as the quality of the product.

INTRODUCTION

Like all complex biological foodstuffs, honey is subject to changes which may partly impair its nutritional and organoleptic properties. Some of these changes occur

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simply with age, whereas others depend upon the methods used in processing and conditions of storage. Variations in water content and acidity are particularly important; e.g., increase in water content with decrease in acidity favours microbial growth.

Within certain limits, water content variability as a hazardous factor would depend upon the number of microorganisms and spores present (Leuveant, 1968; Troller, 1979), but to obviate microbial development 20% moisture should not be exceeded. This safety limit does not seem to be universally respected. In Sardinia, for example, an examination of 26 samples showed that 10 of them had a water content of well over the 20% mark, one indeed reaching 25% (Campus et al., 1983). The law passed in Italy on 22nd October 1982 (following the EEC directive to equalize legislation on honey) does not cover the question of microbial content; nor does it deal adequately with the problem of moisture. Article II, section 2, states «water content: not more than 21%; (but) for heather, clover and strawberry-tree honeys: not more than 23%».

Generally, honey is not a good substrate for the development of microorganisms, but some, for instance osmophilic yeasts and moulds, manage to do so and cause spoiling (Farris et al., 1982; Malan and Ozino-Marletto, 1974; Marletto et al., 1975; Miniria, 1976; Vecchi and Zambonelli, 1979; Yamazaki et al., 1975). Regarding the presence of bacteria, the literature appears to be not very extensive, being limited to a generic count of some sporogenous forms, examining the survival capacities of certain pathogenic species (Hornitzky, 1982; Tysset and Durand, 1973; Tysset et al., 1979), and the detection of: the non-sporing *Gluconobacter* sp. and *Lactobacillus viridescens* (Ruiz-Argueso and Rodrigues-Navarro, 1973), the aerobic spore-forms *Bacillus larvae*, *B. alvei*, *B. thuringiensis*, *B. subtilis*, *B. megaterium* (Borges, 1976; Farris et al., 1982; Tysset and Rousseau, 1981; Vecchi and Zambonelli, 1968); and the anaerobic spore-forms *Clostridium botulinum* (Arnon et al., 1979; Bentler and Frese, 1981; Huhtanen et al., 1981; Midura and Arnon, 1976; Sugiyama et al., 1978). As is well known, ingesting the latter with honey can give rise to botulism particularly in 1 to 6 month-old babies (Aureli and Accorti, 1981; Midura and Arnon, 1976; Pickett et al., 1976).

Thus, microbiological as well as chemical and physical standards are necessary in order to establish satisfactory food-safety regulations for commercial honeys. However, a great deal more information is required on their microbiological composition for legislation to be adequate. It is hoped that the results reported in this paper will help towards this end.

MATERIALS AND METHODS

The 52 samples of honey examined came from different localities in Sardinia, 34 being of multifloral origin and 18 unifloral¹.

Most of them had been stored for 1 year, 8 for 6 months and 1 for 2 years. All were taken from honeys produced in movable frame hives and which had undergone no commercial processing.

The analysis of each sample was carried out by first heating 100g in 300-ml flasks to 80°C and continuously agitating at this temperature for 10 min. Of each heated sample, 10g were then taken and dilutions prepared in sterile physiological saline solution and replicated 3 times. For the *Bacillus* spore count, 1 ml of each dilution was poured into a petri-dish containing nutrient agar (Difco); for the *Clostridium* spore count - Weinzirl method (Weinzirl, 1916) - 1 ml of each dilution was poured into a test-tube containing liquid RCM (Reinforced Clostridial Medium, Oxoid) and heated at 80°C for 2 min to melt the paraffin seal and eliminate any possible oxygen. Counts were effected after incubating all the media at 35°C for 48 - 72 h, the Hoskins MPN (Most Probable Number) method being used for the clostridial spores (Hoskins, 1983). For isolating *Bacillus* cultures, dilutions were streaked onto nutrient-agar dishes; and for *Clostridium* onto RCM-agar dishes incubated anaerobically at 35°C (BBL Gas Pak System). The API 50 CHB system was used to identify *Bacillus* strains, and the methods of Buttiaux (Buttiaux et al., 1975) for *Clostridium* strains.

RESULTS AND DISCUSSION

Table 1 gives the number of spores found, plant origin and storage age for each of the honey samples, 88.4% of which contained bacterial spores. Of these, 86.5% were aerobes and 19.2% anaerobes. In many instances the counts were high, and even discounting the question of deleterious effects, it would seem that extraction and storage procedures were insufficiently circumspect, as we have often seen in many processing centres.

The strains isolated amounted to 187 and are reported in table 2; 162 belonged to the genus *Bacillus* and 25 to *Clostridium*. Six species of the former genus were classified. One of these, *B. alvei* (once considered the presumed agent of European foul brood) was present in 15.3% of the samples. *B. cereus* was present in 48.0% of the samples, and can be a serious danger in honey destined for the food or cosmetic

¹ Melisso-palynological analyses effected by Dr. Angela Melis, Apiculture Laboratory, Institute of Agricultural Entomology, University of Sassari, Italy.

Table 1 - Plant origin, storage age and microbic count for each honey sample
Origine florale, età di conservazione e conta microbica per ciascun campione di miele

Sample	Plant origin	Age (years)	<i>Bacillus</i> (spores/g)	<i>Clostridium</i> (spores/g)
1	<i>Multiflora</i>	1	100	4
2	<i>Multiflora</i>	1	100	
3	<i>Arbutus unedo</i>	1	2	
4	<i>Multiflora</i>	1	4	
5	<i>Arbutus unedo</i>	1	10	
6	<i>Multiflora</i>	1	100	
7	<i>Multiflora</i>	1	1	
8	<i>Multiflora</i>	1	1	
9	<i>Multiflora</i>	1		
10	<i>Erica</i> sp.	1	5	
11	<i>Citrus</i> sp.	1	10	
12	<i>Multiflora</i>	1	1,000	
13	<i>Multiflora</i>	1	1,000	
14	<i>Eucalyptus</i> sp.	1	1,000	4
15	<i>Arbutus unedo</i>	0,5	10	
16	<i>Asphodelus</i> sp.	1	10	4
17	<i>Multiflora</i>	1	10	
18	<i>Arbutus unedo</i>	1	10	
19	<i>Multiflora</i>	1	100	
20	<i>Arbutus unedo</i>	1	100	
21	<i>Multiflora</i>	1	1,000	
22	<i>Multiflora</i>	1	10	
23	<i>Multiflora</i>	1	4	
24	<i>Multiflora</i>	2		
25	<i>Multiflora</i>	1	100	
26	<i>Multiflora</i>	1	100	
27	<i>Arbutus unedo</i>	1		
28	<i>Multiflora</i>	1	10	8
29	<i>Multiflora</i>	1		
30	<i>Multiflora</i>	1		
31	<i>Multiflora</i>	1	100	
32	<i>Multiflora</i>	1	10	
33	<i>Multiflora</i>	1	10	
34	<i>Erica</i> sp.	1	10	
35	<i>Multiflora</i>	1	1,000	
36	<i>Carduus</i>	1	100	
37	<i>Asphodelus</i> sp.	1	100	8
38	<i>Multiflora</i>	1	10	
39	<i>Multiflora</i>	1	10	
40	<i>Multiflora</i>	1		
41	<i>Multiflora</i>	1		8
42	<i>Multiflora</i>	0.5	100	2
43	<i>Erica</i> sp.	0.5	2	
44	<i>Multiflora</i>	0.5	10	
45	<i>Eucalyptus</i> sp.	0.5	100	4
46	<i>Carduus</i> sp.	0.5	10	
47	<i>Asphodelus</i> sp.	0.5	2	2
48	<i>Multiflora</i>	1	100	
49	<i>Multiflora</i>	1	10	
50	<i>Multiflora</i>	0.5	10	2
51	<i>Multiflora</i>	1	10	
52	<i>Multiflora</i>	1	100	

Tab. 2 - Strains isolated in each honey sample. Specie isolate da ciascun campione di miele

Species	Sample																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Genus <i>Bacillus</i>																										
<i>B. cereus</i>	4	3	2	1			1					5		4	1		2		6	2	9		2		2	2
<i>B. licheniformis</i>													1													
<i>B. polymixa</i>												1														1
<i>B. alvei</i>																		2								1
<i>B. macerans</i>							1									1	2	2			1	2				
<i>B. subtilis</i>				1	2	4				2	2	2	2	1	1	1				2					2	3
Genus <i>Clostridium</i>																										
<i>Cl. acetobutylicum</i>																										
<i>Cl. beijerinchii</i>	3													3		2										

(segue) Tab. 2

Species	Sample																									
	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
Genus <i>Bacillus</i>																										
<i>B. cereus</i>					2	2				2	4		2			2			1		4		2		1	
<i>B. licheniformis</i>							1		1			2						3	4		1			1		
<i>B. polymixa</i>								1	3		1				1	1		2				1			1	
<i>B. alvei</i>								1		1					5						1	1			1	
<i>B. macerans</i>							1				1								2		2	4		2	2	
<i>B. subtilis</i>		2			1																			3	3	
Genus <i>Clostridium</i>																										
<i>Cl. acetobutylicum</i>		4									2													2		
<i>Cl. beijerinchii</i>															3	2				2		2				

industries. Also present were *B. subtilis*, *B. licheniformis*, *B. polymixa* and *B. mace-rans*; with the exception of the latter, all are producers of antibiotic substances. The first two are typical food bacteria, especially sugary foodstuffs, while the latter are characteristic of decomposing vegetable matter. The spores of the last two can remain in honey for an indeterminate length of time, but do not cause spoiling. Three species of *Clostridium* were classified and were present in 10 of the samples, namely *Cl. acetobutylicum*, *Cl. beijerinckii* and *Cl. pasteurianum*. While the effect of these species on honey is not known, *Cl. beijerinckii* has been found in spoiled candied fruits; it might also cause anomalies in honey.

Further experiments are envisaged to individualize each strain and verify if they can develop during storage, or in conditions where processing involves $A_w > 0.9$.

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