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Original article

Behavior of an enterotoxigenic *Staphylococcus aureus* strain during manufacture of the Moroccan fermented milk "Iben"

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Summary — Two lots of Moroccan Iben (skimmed fermented milk) were prepared from raw milk inoculated with 2 levels of an enterotoxigenic *Staphylococcus aureus* strain (10^3 and 10^5/ml; type C toxin) and spontaneous fermentation at room temperature (24–25 °C) for 72 h. With an inoculum level of 1 x 10^3/ml, *S aureus* reached a maximum of 7.2 and 8.3 x 10^4/ml within 24 h with no accumulation of thermonuclease (TNase). With the higher initial inoculum of 1 x 10^5/ml, *S aureus* attained a maximum level of 1.8 and 3.4 x 10^7/ml with accumulation of detectable TNase and enterotoxin within 24 h. *S aureus* level declined between 24 and 72 h in Iben, whereas both TNase and enterotoxin C persisted. TNase was first detected between 8 and 12 h with an *S aureus* level ≈ 2 x 10^6/ml and no enterotoxin C, whereas both TNase and toxin were detected later (between 12 and 24 h) with an *S aureus* level of 4.9 x 10^6/ml in one lot and 3.4 x 10^7/ml in the other. Control of initial *S aureus* level to 10^3/ml in milk and detection of TNase as a screening test for enterotoxin presence in Iben should help to reduce the public health hazards of staphylococcal food poisoning.

Iben / *S aureus* / thermonuclease / enterotoxin

Résumé — Comportement d'une souche de *Staphylococcus aureus* entérotoxinogène au cours de l'élaboration du lait fermenté marocain «Iben». *Deux lots de Iben marocain (lait fermenté écrémé) sont préparés à partir d'un lait cru inoculé avec 2 taux différents (10^3 et 10^5/ml) d'une souche de* Staphylococcus aureus *productrice d'entérotoxine C. La fermentation spontanée est conduite à température ambiante (24–25 °C) pendant 72 h. Avec un taux initial de 1 x 10^3/ml,* S aureus *atteint son maximum (7,2 et 8,3 x 10^4/ml) au bout de 24 h sans accumulation de thermonuclease (TNase) dans les 2 lots. Avec un taux initial de 1 x 10^5/ml,* S aureus *atteint 1,8 et 3,4 x 10^7/ml après 24 h avec production de TNase et d'entérotoxine C dans les 2 lots de lait. Entre 24 et 72 h, les taux de* S aureus *diminuent dans le Iben alors que la TNase et l'entérotoxine persistent. La TNase est détectée bien avant l'entérotoxine (entre 8 et 12 h) lorsque le taux de* S aureus *est voisin de 2,0 x 10^6/ml. La détection simultanée de la TNase et de l'entérotoxine est obtenue entre 12 et 24 h avec un taux de* S aureus *de 4,9 x 10^6/ml dans un lot et 3,4 x 10^7/ml dans l'autre lot. L'utilisation de lait contenant moins de 10^3* S aureus*/ml et la recherche de la TNase pour sonder la présence éventuelle d'entérotoxine dans le Iben sont des mesures qui peuvent réduire les risques d'entérotoxicose staphylococcique.*

Iben* / S aureus / *thermoculéase* / *entérotoxine

INTRODUCTION

Staphylococcus aureus may be found in raw milk originating either from cows or humans (hand milking) and these can multiply during storage, transport and processing such as in dairy products made from raw milk (Sharpe *et al,* 1965; Abo-Elnaga, 1972; Minor and Marth, 1972; Naguib *et al,* 1979; Dos Santos *et al,* 1981; Holmberg and Blake, 1984; Batish and Chander, 1987; Hamama, 1989). Further contamination from human sources may also occur at the processor level in dairy shops that manufacture and sell traditional Moroccan dairy products, namely lben (skimmed fermented milk), jben (traditional fresh cheese), raib (fermented milk) and farm butter (Hamama, 1989). Enterotoxigenic *S aureus* were found in raw milk at a level of 10^4–10^5/ml and also in traditional dairy products (10^5–10^6/ml or g). Although data regarding the incidence of staphylococcal intoxications from these products are not available, some of these products, particularly lben (5 market samples of 30) showed preformed enterotoxin C (Hamama, 1989). No other type of staphylococcal enterotoxin was found in this product. Some studies indicate that in general *S aureus* is a poor competitor with raw milk microflora (Troller and Frazier, 1963; Radish *et al,* 1967; Halpin-Dohnalek and Marth, 1989) and that in high count raw milk it may grow poorly with no enterotoxin produced (Donnelly *et al,* 1968; Tatini *et al,* 1971). Moroccan raw milk used for making lben is usually of high total count (10^6–10^7/ml) and fermentation takes place at room temperature (15–25 °C) depending on the season. This may not favor growth of *S aureus* and production of enterotoxin in such conditions. However, the behavior of *S aureus* appears to depend upon many other factors such as the nature of the competing microflora, *S aureus* initial level, pH and temperature of fermentation. Until now, no information has been available on the fate of enterotoxigenic *S aureus* during lben making. Therefore, the aim of this work was to study the behavior of an enterotoxigenic *S aureus* strain with 2 initial levels during lben manufacture relative to growth and production of thermonuclease (TNase) and enterotoxin.

MATERIALS AND METHODS

S aureus *culture*

S aureus strain L30 (API Staph System code 7740, Analytab Products, Plainview, NY, USA) previously isolated from lben (Hamama, 1989) and which produces enterotoxin C was used in this study. This strain produces 11.5 µg TNase/ml (in brain heart infusion (BHI) broth after 24 h at 37 °C) which approximates the average level of TNase produced by enterotoxigenic strains isolated from Moroccan traditional dairy products (Hamama, 1987). A 24-h culture of this organism in BHI broth at 37 °C was obtained and used to make different dilutions. The optical density (OD, 600 nm) was measured with a spectrophotometer (Spectronic 20, Bausch and Lomb, USA) in order to prepare a standard equation of the type $Y = aX + b$ where Y stands for log *S aureus* count and X for the corresponding OD. The equation obtained had the following formula: $Y = 4.70\ X + 7.23$ and was used to make the appropriate dilutions of *S aureus* culture to obtain the desired inocula of 1×10^3 and 1×10^5 *S aureus*/ml of raw milk.

Inoculation of raw milk

Two lots of 9 l of raw milk were obtained from the experimental farm of the Institut Agronomique et Vétérinaire Hassan II (Moghrane, Gharb) in sterilized glass containers. Each lot was divided into 3 portions of 3 l each. Two portions were inoculated with the appropriate dilution of a 24-h culture of *S aureus* L30 to obtain

inoculum levels of 1 x 10^3 and 1 x 10^5/ml of raw milk. The third non inoculated portion was used as control. The inoculated and control milks were held at room temperature (24–25 °C) to allow spontaneous fermentation as is done by the dairy shops to make Iben.

Sampling and analyses

Though coagulation of raw milk usually occurred after 48 h of incubation at room temperature, Iben was made from this fermented milk after an additional 24 h (total of 72 h). Coagula were churned for 20 to 30 min in half-filled glass containers. A volume of approximately 10% warm sterile water was added during churning to help aggregate butter grains at the surface, which were then removed using a sterile scoop.

Samples at 0, 4, 8, 12, 24, 48, 72, 96 and 120 h following *S aureus* inoculation were examined for pH, total aerobic count, *S aureus* count and presence of TNase and enterotoxin.

pH measurement

Sample pH was determined using a pH meter apparatus (E-520 Metrhom Herizan, Switzerland).

Microbial counts

Standard plate count (SPC) was obtained by pour-plating appropriate sample dilutions in plate count agar (PCA) plates and incubating for 48 h at 32 °C (Messer *et al,* 1985).

S aureus count was determined by surface plating of appropriate sample dilutions on Baird-Parker agar plates and incubating for 48 h at 37°C (Tatini *et al,* 1984).

Thermonuclease testing

Thermonuclease was extracted using a sample of 40 ml of raw milk, coagulum or Iben adjusted to pH 4.5 (Tatini *et al,* 1976) when necessary with a solution of 3 N HCl. Samples were then centrifuged at 10 000 rpm for 20 min using a RC-2 Sorvall refrigerated centrifuge (Dupont Instruments, USA). According to the simplified procedure of Ibrahim (1981), the supernatant was not concentrated. The latter was heated for 60 min at 100 °C (Tatini *et al,* 1984) to eliminate non staphylococcal TNase and allowed to cool. Twenty-five µl of extract was used to fill a 5-mm well cut into a Toluidine blue DNA agar plate (TB-DNA). To prevent eventual false positive reactions due to interaction of the acidic extract with the dye, another 25 µl of extract was placed in similar well cut into a DNA agar plate (without the dye). The DNA media (with and without the dye) were prepared as described by Kamman and Tatini (1977). After 4 h incubation at 50 °C, as recommended by Ibrahim (1981), the TB-DNA plates were observed for presence of a pink halo and the DNA plates for a clear halo around the wells. In the latter instance, TNase reaction was noted by flooding the DNA agar plates with 1 N HCl. Only samples showing specific and distinctive reactions (haloes extending 1 mm beyond the well) on both TB-DNA and DNA plates were considered positive.

Staphylococcal enterotoxin detection

The presence of staphylococcal enterotoxin C (SEC) was detected with staphylococcal enterotoxin reversed passive latex agglutination (SET-RPLA) test kit (Lot 34701-Oxoid Limited, Basingstoke, UK) for enterotoxin detection. The Oxoid kit recommended method for enterotoxin extraction based on a 1:2 dilution with saline solution and centrifugation was not followed. To avoid any dilution of toxin present, we instead used the supernatant (without prior boiling) from TNase testing as extract. The latter also had the advantage of being acid-treated.

RESULTS AND DISCUSSION

Data on the 2 lots of Iben made from raw milk inoculated with 2 initial levels of enterotoxigenic *S aureus* strain L30 are presented in tables I and II. As can be seen from the tables, there was good fermentation in the 2 lots and the pH reached 4.5–4.7 after 48 h. With 10^3 *S aureus* inoculum in both lots of Iben, *S aureus* reached a maximum population of 7.2 and 8.3 x

10^4/ml and subsequently declined with no accumulation of TNase. Uninoculated milks showed no detectable *S aureus* (< 10/ml) throughout the experiment. None of the samples (inoculated or not with *S aureus*) showed TNase, and therefore, were not expected to contain enterotoxin and thus were not tested for its presence.

With 10^5/ml initial inoculum, *S aureus* attained a maximum population of 1.8 and 3.4 x 10^7/ml with the corresponding samples being positive for TNase and enterotoxin. It can also be seen from these data that TNase was detected before enterotoxin in each lot. Samples with 1.8–2.2 x 10^6 *S aureus*/ml were positive for TNase but

Table I. Growth and TNase production by *S aureus* during manufacture of lben from raw milk. *Croissance et production de TNase par* S aureus *au cours de la préparation du lben à partir du lait cru.*

Sampling time (h)	*Lot*	*Milk inoculated with 1 x 10^3* S aureus*/ml*			
		pH	*SPC (1 x 10^6/ml)*	S aureus *count (1 x 10^3/ml)*	*TNase reaction*
Raw milk					
0	1	6.6	3.5	2.6	–
	2	6.7	1.3	1.6	–
4	1	6.6	8.8	4.5	–
	2	6.7	2.2	3.3	–
8	1	6.4	29.0	21.0	–
	2	6.6	14.0	15.0	–
12	1	6.1	73.0	56.0	–
	2	6.4	31.0	46.0	–
24	1	5.3	213.0	72.0	–
	2	5.8	230.0	83.0	–
Coagulum					
48	1	4.5	870.0	6.8	–
	2	4.6	1600.0	14.0	–
Lben					
72	1	4.4	1200.0	3.1	–
	2	4.5	2800.0	11.0	–
96	1	4.1	3300.0	< 0.03	–
	2	4.2	3600.0	0.1	–
120	1	ND	ND	ND	–
	2	4.0	2300.0	< 0.03	–

SPC: Standard plate count; ND: Not determined.

Table II. Growth and TNase and enterotoxin production by *S aureus* during manufacture of Iben from raw milk.
Croissance et production de la TNase et de l'entérotoxine par S aureus *au cours de la préparation du Iben à partir du lait cru.*

Sampling time (h)	*Lot*	*Milk inoculated with 1 x 10^5* S aureus*/ml*				
		pH	*SPC (1 x 10^6/ml)*	S aureus *count (1 x 10^5/ml)*	*TNase reaction*	*SEC*
Raw milk						
0	1	6.6	3.8	2.2	–	ND
	2	6.7	1.8	1.4	–	ND
4	1	6.6	9.3	3.6	–	ND
	2	6.7	2.6	4.7	–	ND
8	1	6.4	33.3	8.0	–	ND
	2	6.6	19.0	18.0	+	–
12	1	6.1	74.0	22.0	+	–
	2	6.4	37.0	49.0	+	+
24	1	5.2	250.0	340.0	+	+
	2	5.8	310.0	176.0	+	+
Coagulum						
48	1	4.5	900.0	120.0	+	+
	2	4.7	2200.0	84.0	+	+
Lben						
72	1	4.4	1600.0	98.0	+	+
	2	4.6	3400.0	61.0	+	+
96	1	4.0	3400.0	0.02	+	+
	2	4.2	3900.0	0.08	+	+
120	1	3.8	2700.0	< 0.0003	+	+
	2	4.0	2600.0	< 0.0003	+	+

SPC: Standard plate count; SEC: staphylococcal enterotoxin C (RPLA test); ND: not determined.

not for enterotoxin, while those at higher levels of *S aureus* (4.9 x 10^6 to 3.4 x 10^7/ml) were positive for both TNase and enterotoxin C. These data indicate that high initial levels of *S aureus* (10^5/ml) in raw milk can reach toxigenic levels in Iben made even when the total aerobic count of raw milk was high (5 x 10^6–7.5 x 10^7/ml). This is in contrast to the studies of Donnelly *et al* (1968) and Tatini *et al* (1971) who reported lack of *S aureus* growth and/or production of enterotoxin A in high count

raw milk. However, this difference may be due to the nature of the microflora present in the raw milks used. In our study, the initial flora was mesophilic while in the previous works cited, it was predominantly psychrotrophic.

The data obtained in this study confirm the earlier reports of others who noted *S aureus* levels of ≥ 10^6/ml for detection of TNase (Tatini, 1981) and higher levels of 10^7/ml for enterotoxin detection (Donnelly *et al,* 1968; Tatini *et al,* 1971, 1976) with the exception that in the present experiment TNase was detected even without concentrating the supernatant; this was compensated by the use of an increased test sample size (25 µl in this study instead of 5 µl for the method with concentration). The method used in this study for extraction and detection of TNase from milk and Iben and which is a compilation of several previous procedures (Tatini *et al,* 1976, 1984; Ibrahim, 1981) was already used to screen the presence of enterotoxin in raw milk and traditional Moroccan dairy products (Hamama, 1989). This method was found to be sufficiently sensitive to detect TNase prior to accumulation of enterotoxin amounts detectable by the RPLA kit.

Data presented here indicate that raw milk containing high enterotoxigenic *S aureus* count (10^5/ml) can result in enterotoxin in Iben made with spontaneous fermentation, and such growth can be detected by TNase before the development of enterotoxin C. Several other studies have been conducted in the past on the behavior of *S aureus* in different types of dairy products (Miller and Ledford, 1977; Dos Santos and Genigeorgis, 1981; Ahmed *et al,* 1983; Otero *et al,* 1988). These studies reported contradictory results regarding the minimal levels of *S aureus* needed before accumulation of detectable amounts of TNase. Tatini *et al* (1976) noted detectable levels of TNase in milk and whey when the *S aureus* count was 5 x 10^5/ml and enterotoxin when the latter reached 5 x 10^6–1 x 10^7/ml, while Miller and Ledford (1977) detected TNase in Cheddar cheese whey but not enterotoxin at 1.7 x 10^7 *S aureus*/ml. When examining potential growth of staphylococ-

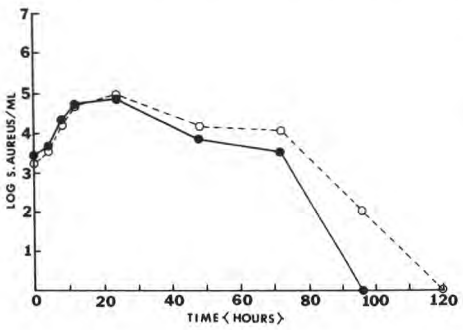

Fig 1. Growth of *S aureus* in raw milk during Iben making (inoculum level : 1 x 10^3/ml) (●—● Lot 1, O– – –O Lot 2).
Croissance de S aureus *dans le lait cru au cours de la préparation du Iben (taux d'inoculation : 1 x 10^3/ml) (●—● Lot 1, O– – –O Lot 2).*

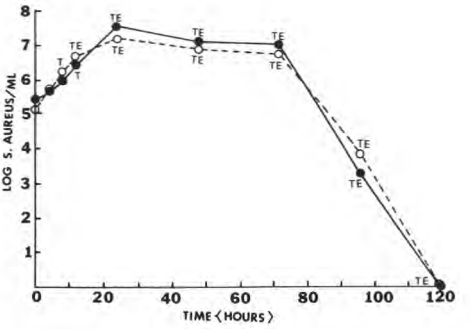

Fig 2. Growth of *S aureus* in raw milk during Iben making (inoculum level : 1 x 10^5/ml) (●—● Lot 1, O– – –O Lot 2, T : TNase presence; TE : TNase and enterotoxin presence).
Croissance de S aureus *dans le lait cru au cours de la préparation du Iben (taux d'inoculation : 1 x 10^5/ml) (●—● Lot 1, O– – –O Lot 2, T : présence de TNase; TE : présence de TNase et d'entérotoxine).*

ci in Brazilian Minase cheese whey, Dos Santos and Genigeorgis (1981) detected TNase only in samples exceeding 10^7/ml *S aureus* count. In Domiati cheese whey, Ahmed *et al* (1983) detected TNase and enterotoxin A when the number of *S aureus* was 1.6 x 10^8/ml. Otero *et al* (1988) studied the behavior of 2 *S aureus* strains producing enterotoxin C during the manufacture and storage of Burgos cheese. They found that the TNase test is of a limited value in assessing staphylococcal growth in this type of cheese. With *S aureus* FRI 361 strain (low TNase producer: 0.8 µl/ml), this organism reached 10^7/g of cheese without amounts of detectable TNase while with strain FRI 137 (high TNase producer: 20.91 µg/ml), TNase was detected when *S aureus* attained 6.7 x 10^6/g.

It seems then that the production of TNase is mainly related to the nature of the *S aureus* strain (low, average or high TNase producer) and to the different factors influencing *S aureus* growth (temperature and pH of the substrate, initial level of *S aureus*, type and level of competing flora, etc). Therefore, the TNase testing might not have the same usefulness in all food products and under all conditions. The findings reported in this study are then valuable only for this type of fermented milk and for the particular *S aureus* strain used in this experiment although the origin of Iben and its particularities (production of SEC and average level of TNase) make it suitable enough to assess the behavior of enterotoxigenic *S aureus* in Iben.

Data of this experiment showed that initial levels of *S aureus* $\leq 10^3$/ml are not expected to result in enterotoxin C in Iben. Subsequently, measures such as control of staphylococcal mastitis in cows and of human contamination during milking are essential to reduce enterotoxigenic *S aureus* to $\leq 10^3$/ml in raw milk. However, such measures may not be easy to apply at the present time in Morocco. Since both raw milk and Iben showed presence of enterotoxigenic *S aureus* and also enterotoxin C at levels that can cause food intoxication in Iben made from raw milk (Hamama, 1989), it is essential to favor a very active fermentation of raw milk so that its pH decreases rapidly to < 5.5 before *S aureus* attains hazardous levels (> 10^6/ml) even when the *S aureus* initial count is 10^5/ml. Probably, addition of 2–3% of good quality Iben to raw milk as a first step in Iben manufacture may help in the control of excessive *S aureus* growth. This alternative deserves to be experimented with in future.

With respect to the TNase test, data presented here suggest that this rapid test should be considered as a valuable tool in screening Iben for staphylococcal enterotoxin.

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REFERENCES

Abo-Elnaga IG (1972) Staphylokokkenvermehrung in gesalzener Rohmilch für die Herstellung von Salzlakenweisskäse. *Milchwissenschaft* 27, 583-586

Ahmed AAH, Moustafa MK, Marth EH (1983) Growth and enterotoxin production by *Staphylococcus aureus* in whey from the manufacture of Domiati cheese. *J Food Prot* 46, 235-237

Batish VK, Chander H (1987) Occurrence of *Staphylococcus aureus* and their preformed enterotoxins in frozen dairy desserts. *Aust J Dairy Technol* 42, 22-24

Donnelly CB, Leslie JE, Black LA (1968) Production of enterotoxin A in milk. *Appl Microbiol* 16, 917-924

Dos Santos EC, Genigeorgis C (1981) Potential for presence and growth of staphylococci in Brazilian Minas cheese whey. *J Food Prot* 44, 185-188

Dos Santos EC, Genigeorgis C, Farver TB (1981) Prevalence of *Staphylococcus aureus* in raw and pasteurized milk used for commercial manufacturing of Brazilian Minas cheese. *J Food Prot* 44, 172-176

Halpin-Dohnalek MI, Marth EH (1989) *Staphylococcus aureus*: production of extracellular compounds and behavior in foods. A review. *J Food Prot* 52, 267-282

Hamama A (1987) Thermonuclease and enterotoxin and biotyping of staphylococci isolated from Moroccan dairy products. *Lait* 67, 403-411

Hamama A (1989) Studies on the hygienic quality of Moroccan traditional dairy products. PhD Thesis, Univ Minnesota, St Paul, MN, USA

Holmberg SD, Blake PA (1984) Staphylococcal food poisoning in the United States. New facts and old misconceptions. *J Am Med Assoc* 251, 487-489

Ibrahim GF (1981) A simple sensitive method for determining staphylococcal thermonuclease in cheese. *J Appl Bacteriol* 51, 307-312

Kamman JF, Tatini SR (1977) Optimal conditions for assay of staphylococcal nuclease. *J Food Sci* 42, 421-424

Messer JW, Behney HM, Leudecke LO (1985) Microbiological count methods. *In: The Standard Methods for the Examinations of Dairy Products* (Richardson GH, ed) Am Publ Health Assoc Washington, DC, 15th edn, ch 6, 327-404

Miller JS, Ledford RA (1977) Growth inhibition of *Staphylococcus aureus* in Cheddar cheese whey. *J Dairy Sci* 60, 1689-1692

Minor TE, Marth EH (1972) *Staphylococcus aureus* and staphylococcal food intoxication. A review. III: Staphylococci in dairy foods. *J Milk Food Technol* 35, 77-82

Naguib MM, Nour MA, Noaman AA (1979) Survival of *Staphylococcus aureus* in Ras cheese. *Arch Lebensmittelhyg* 30, 227-228

Otero A, Garcia MC, Garcia ML, Prieto M, Moreno B (1988) Behaviour of *Staphylococcus aureus* strains, producers of enterotoxins C1 or C2, during the manufacture and storage of Burgos cheese. *J Appl Bacteriol* 64, 117-122

Radish R, Sandine WE, Elliker PR (1967) Inhibition of *Staphylococcus aureus* by *Streptococcus diacetylactis* in milk and cream fillings. *J Dairy Sci* 52, 880 (Abstr)

Sharpe ME, Fewins BG, Reiter B, Cuthbert WA (1965) A survey of the incidence of coagulase-positive staphylococci in market milk and cheese in England and Wales. *J Dairy Res* 32, 187-192

Tatini SR (1981) Thermonuclease as an indicator of staphylococcal enterotoxins in foods. *In: Antinutrients and Natural Toxicants in Foods* (Ory RL, ed) Food and Nutrition Press, Wesport, CT, 53-75

Tatini SR, Jezeski JJ, Olson JC, Casman EP (1971) Factors influencing the production of staphylococcal enterotoxin A in milk. *J Dairy Sci* 54, 312-320

Tatini SR, Cords BR, Gramoli J (1976) Screening for staphylococcal enterotoxin in food. *Food Technol* 40, 64-76

Tatini SR, Hoover DG, Lachica RVF (1984) Methods for the isolation and enumeration of *Staphylococcus aureus*. *In: The Compendium of Methods for the Microbiological Examination of Foods* (Speck ML, ed) Am Publ Health Assoc, Washington, DC, 411-427

Troller JA, Frazier WC (1963) Repression of *Staphylococcus aureus* by food bacteria. I: Effect of environmental factors on inhibition. *Appl Microbiol* 11, 11-14