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The microbial flora of sugary kefir grain (the gingerbeer plant): biosynthesis of the grain from *Lactobacillus hilgardii* producing a polysaccharide gel

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Introduction

The word 'kefir' commonly indicates a milky beverage whose fermentation has been induced by small, white, lobed, resilient, cauliflower-shaped masses named 'kefir grains', supposedly found only in the Caucasus. The microbiological population is firmly embedded in these grains. However, there is another beverage, made from sugar and water with figs and lemon added, whose fermentation is induced by grains of a different type which are transparent, mucilaginous, but less resilient. These grains have been given various names such as Gingerbeer plants (Ward 1892; Hesseltine 1965), California bees (Kleber 1921), Tibis grains or Tibi-complex (Lutz 1899; Horisberger 1969; Moinas *et al.* 1980). The Tibis grains are known to originate from a Mexican cactus (Opuntia) where they were taken off the leaves. However, uncertainty remains about the origin of the other grains. Beijerinck (1889) linked the 'kefir grains' to the gingerbeer plants that English soldiers brought back from the Crimean war in 1855. In France, the transparent grains, which are the subject of the present study, are named 'kefir grains' (Vayssier 1978) and they will be called *sugary kefir grains* in order to differentiate them from the grains fermenting milk.

The particular properties of these different grains and their products are:

(a) They increase in size and divide in the growth medium resulting in a greater number of cells being immobilized.
(b) Regular fermentation occurs over a long period if the culture conditions are identical. Tibis grains used by Moinas *et al.* (1980) have been sub-cultured for 10 years, showing a steady association between the different strains.
(c) This stability is not lost by the lack of aseptic culture conditions; the reason for this is unknown, although 'the material responsible for embedding the organisms would

appear to be an important factor in sustaining a favourable and specific ecological niche' (Wood & Hodge 1985). It would appear that some pH-dependent, inhibitory compound (Collins-Thompson *et al.* 1983) or some antagonistic substance is operating.
(d) The nutritional and medical qualities of the food fermented by yeast-lactic acid bacteria associations have been underlined (Wood & Hodge 1985), but further investigations are needed to improve the knowledge of such interesting interactions. It is remarkable that the same qualities have been emphasized for the milky kefir grains (La Rivière *et al.* 1963, 1967).

The aims of the present study were to demonstrate the numerical importance of each group of microorganisms in household kefir grains, and to identify the different species of bacteria and yeasts present, in order to select the strains of polysaccharide-producing, and especially the gel-forming, species needed to carry out a controlled biosynthesis of new grains. The manufacture of the new grains represents an alternative way of using alginate or carrageenan gels for the immobilization of microorganisms, with the particular advantage of increasing the volume of fixed cells, using an economical amount of sucrose solution.

Materials and methods

The household sugary kefir grains originated from the Angers area of France, and were propagated at room temperature by daily transfer in tap water-sugar solution (60 g/litre).

Enumeration of bacteria and yeasts
From household grains, 20 g of wet grains in 180 ml of sterile distilled water were disrupted in a homogenizer (Waring Blender) for 5 min. The first dilution was used to detect the possible contaminants (coliforms, staphylococci, *Pseudomonas*, anaerobic sulphite reducers) by the use of desoxycholate-lactose (Institut Pasteur Production (IPP)) and Baird Parker (IPP), cetrimid (Merck) and TSN (Merck) agar, respectively. Faecal streptococci were detected on Barnes agar (Merck) then on Rothe (IPP) and Litsky (IPP) broths.

Further dilutions were plated on the following agars: Bacto Plate Count Agar (PCA, Difco) + 1% (w/v) skimmed milk, PCA + 1% skimmed milk + 0.03% (w/v) cycloheximide, MRS (Merck) and Elliker (Difco), RMW (Merck), M17 (Difco), MSE + 0.075% (w/v) of sodium azothydrate (Mayeux *et al.* 1962), Potato Dextrose Agar (PDA, Merck), for the counting of total microorganisms, total bacteria, lactic acid bacteria, lactobacilli, lactic streptococci, polysaccharide-forming Gram-positive bacteria, and yeasts respectively. The temperatures and times of incubation were: 23, 30, 37°C for 48 or 72 h for the lactic acid bacteria, and 22°C for 72 h for the yeasts. A gas-generating kit (Oxoid, BR56) was used in a gaspack jar to incubate the lactic acid bacteria microaerobically.

From newly synthesized grains, 5 g were crushed for 2 min in 10 ml of Ringer's Solution in the presence of 500 U of dextranase grade III (Sigma), left at 37°C for 1.5 h for a partial enzymatic hydrolysis, then crushed again (Ultraturrax, 3 min) in a quantity of Ringer's solution giving the first dilution. Successive dilutions were plated on MRS agar and the incubation performed at 30°C for 3 days.

Selection and Identification

Yeasts. All the colonies morphologically different from each other on PDA were picked off and purified by streaking on the same medium. Ascospore formation was tested on malt extract agar and on sodium acetate (0.4% w/v) medium, and the filamentation test by the method of Dalmau (1929). The capacity to ferment sugars was determined in yeast extract (0.5% w/v) broth with 2% (w/v) sugar, and the assimilation in Bacto Yeast Nitrogen base (Difco) medium. The use of nitrate and hydroxylamine chlorhydrate was tested in Bacto Yeast Carbon base (Difco) medium; the cycloheximide resistance was determined in the same medium with 100 mg/litre of this substance. Arbutin (5% w/v) hydrolysis was carried out in tubes with yeast extract agar (0.3% w/v) and ferric ammonium citrate (two drops of a 1% w/v) solution).

Lactic acid bacteria. The first method was to pick up the colonies which were morphologically different on PCA/milk medium; the second method was by taking more than 50 colonies at random from PCA/milk/cycloheximide medium. The colonies were purified on the same agar, and 50 Gram-positive and catalase-negative bacteria were retained. The lactobacilli and *Leuconostoc* ssp. were identified by the API 50CH system (API, La Balme les Grottes, 38390 Montalieu Vercieu, France), and with the following tests: gas formation from glucose in Gibson & Abdel Malek (1948) medium; arginine hydrolysis in Moeller broth (1955); minimal and maximal growth temperature in MRS broth; L- and D-lactate content in MRS broth by the Boehringer Mannheim kit, to distinguish the subspecies *L. casei* spp. *casei* from *L. casei pseudoplantarum*. The lactic streptococci were submitted to the following tests: gas formation (as above), acetoin production and sodium chloride resistance in skimmed milk (+ 4% w/v NaCl).

Classifications

Lactic acid bacteria were classified according to the criteria listed in *Bergey's Manual of Systematic Bacteriology* (1986), and the yeasts according to Kreger van Rij (1984).

Polysaccharide gel production for transmission electron microscopy (TEM)

The *Lactobacillus hilgardii* variant polysaccharide producer isolated in this study was cultured in a flask of MSE broth at 30°C. After 5 days, the gel formed at the bottom of the flask was removed and pieces were fixed in 3% glutaraldehyde cacodylate buffer (0.2 M, pH 7.4) for 1 h. The samples were stained by ruthenium red (0.04%, w/v) (Luft 1971) in 1% osmic acid/0.1 M cacodylate buffer for 3 h (20°C), washed in the same buffer, dehydrated through a series of ethanol solutions of increasing concentration, embedded in Epon resin, sectioned on a Reichert microtome and contrasted by a uranyl acetate-lead citrate solution. The sections were examined in a JEOL 100B electron microscope at an accelerating voltage of 80 kV.

Scanning electron microscopy (SEM)

Pieces of household kefir grains were fixed as above for 4 h, washed twice in the cacodylate buffer, freeze-dried, gold coated and viewed through a JEOL 35C scanning electron microscope at an accelerating voltage of 20 kV.

Polysaccharide gel production for the biosynthesis of new grains
MSE petri dishes were spread with 4 drops of an MRS broth culture of *L. hilgardii* (24 h at 30°C) and incubated at 30°C. After 4 days, the gel formed on the surface and embedding the bacteria was scraped off and placed for growing in sucrose solution (10% w/v) with yeast extract (0.25% w/v) and magnesium sulphate (0.02% w/v), pH 6.5 (SY solution). The yield of dry polysaccharide formed from the glucose was calculated from the weight of the grains, containing 5% of adsorbed water and 11.5% of dry matter, 100 g of sucrose giving 47 g of glucose by hydrolysis. Since the dry matter of the grains contained about 98% of polysaccharide (Pidoux *et al.* 1988), the grains have been assimilated to a wet polysaccharide embedding bacteria.

Results

Viable counts
The PCA/milk medium gave the highest number of microorganisms at 23°C (Table 1). The kefir grain microflora appeared mesophilic, growing better at 23 or 25°C than at

Table 1 Colony counts of bacteria and yeasts from sugary kefir grains

	Incubation conditions		
Medium*	Time (h)	Temperature (°C)	Viable count (c.f.u./g)
PCA + 1% milk	72	37	$>6 \times 10^8$ bacteria and yeasts
		23	$>2 \times 10^9$ bacteria and yeasts
PCA + 1% milk + 0.03% cycloheximide	48	30	$>0.6 \times 10^9$ bacteria and resistant yeasts
MRS (pH = 6.5)	72	37	$>5 \times 10^6$ lactic acid bacteria
	72	23	$>5 \times 10^8$ lactic acid bacteria
Elliker (pH = 6.8)	72	30	$>3 \times 10^9$ lactic acid bacteria
		23	$>1 \times 10^9$ lactic acid bacteria
RMW (pH = 5.4)	72	37	$>4 \times 10^6$ lactobacilli
		23	$>7 \times 10^8$ lactobacilli
M17	72	30	$>3 \times 10^7$ lactic streptococci
MSE + 0.075% azide	48	30	$>6 \times 10^7$ polysaccharide-producing Gram-positive bacteria
PDA	72	22	$>1 \times 10^8$ yeasts
Desoxycholate 1% lactose	24	37	$>2 \times 10^2$ coliforms
Baird Parker	48	44	$<10^2$
		37	$<10^2$
Cetrimid agar	24	37	$<10^2$
TSN	24	37	$<10^2$
Barnes → Rothe → Litsky	48	37	positive presence of faecal streptococci

* See Materials and methods section.

37°C. The lactic acid bacteria constituted the most important group within the microflora, in the following order of numerical importance: lactobacilli, *Leuconostoc*, lactic streptococci. The yeasts accounted for about 3% of the complete flora.

Few coliforms and faecal streptococci were detectable; no staphylococci, pseudomonads or anaerobic sulphite reducers were found.

Identification and percentages of the lactic acid bacteria

Several species, isolated according to the different morphological aspect of their colonies, were not found by examining colonies at random: *L casei* ssp. *rhamnosus* (growing at 45°C), *L. plantarum*, *S. lactis* and *S. cremoris*. Within the statistical variations, this indicates that these species are <6% of the complete flora (Table 2).

Table 2 Lactic acid bacteria and yeasts in sugary kefir grains and percentages of the bacteria species

	Species isolated on		
	PDA (yeasts)	PCA + 1% milk (Lactic acid bacteria)	Percent of lactic acid bacteria*
	Zygosaccharomyces florentinus	*Lactobacillus hilgardii*	30%
	Torulaspora pretoriensis	*Lactobacillus casei* ssp. *casei*	42%
	Kloeckera apiculata	*Lactobacillus casei* ssp. *rhamnosus*	<6%**
	Candida lambica	*Lactobacillus plantarum*	<6%**
	Candida valida	*Leuconostoc mesenteroides* ssp. *dextranicum*	28%
		Streptococcus lactis	<6%**
		Streptococcus cremoris	<6%**
Number of colonies isolated for identification	11	18	
Number of different strains	5	7	

* Isolated from a random selection of 50 purified Gram-positive, catalase-positive strains on PCA + 1% milk + 0.03% cycloheximide.

** These species not found among the 50 colonies of lactic acid bacteria statistically <6% of the total.

Lactobacillus casei was the most abundant species, identified by the random procedure (42%), fermenting the tagatose (and aldonitol, *L. casei* ssp. *casei*) and growing in the high concentration of sucrose of the MSE medium. Microscopically, the bacilli appeared in long curved chains. In MRS broth they formed a white, heavy sediment making a clear supernatant. No DL-strain, i.e. producing DL-lactate, was among those tested and therefore the majority of the colonies isolated had to be assigned to *L. casei* ssp. *casei*, some of them being lactose negative.

Lactobacillus hilgardii was a widely represented species (30%), growing at 15 to 39°C. Two variants were previously found on MSE agar (data to be published) with

only one of them producing a polysaccharide which formed a gel after 4 days. These two variants gave the same API 50CH profile with a slow utilization of sucrose. The other sugars fermented were: arabinose, xylose, ribose, galactose, glucose, fructose, maltose and gluconate. Despite the fermentation of arabinose, this strain was identified as *L. hilgardii* because of the non-fermentation of melibiose and principally the DNA/DNA homology with *L. hilgardii* NCDO 264 (results to be published). Like the *L. casei* strains, they formed a sediment in MSE broth.

Leuconostoc mesenteroides ssp. *dextranicum* was very often found among the lactic acid bacteria (28%). The strains isolated produced slime on MSE agar but these polysaccharides never became a gel although a sediment of insoluble polymer was sometimes observed in MSE broth. They did not ferment arabinose and produced only D-lactate.

Identification of the yeasts

Pseudomycelia were produced by the two *Candida* identified, but not by the other species of yeast (Table 2). These identifications were confirmed by the Centraal Bureau voor Schimmelcultures of Baarn (Holland).

Observations by scanning and transmission electron microscopy

Whereas the inner part of the grain showed a low content of microorganisms, as seen by SEM examination, the outer was covered by a network of pseudomycelia bearing blastospores and stuck together or to colonies of bacteria (Fig. 1c, d). These pseudomycelia can be ascribed to the *Candida* described in Table 2. The other species of yeast did not appear to be attached to the grain but only retained in the spaces inside the polysaccharidic matrix or in the cracks formed by the gas pressure increasing during fermentation.

The *L. hilgardii* variant forming gelling colonies was seen to be partially covered by a patch of substance (Fig. 2) identified as a polysaccharidic capsule by the ruthenium red staining (Fig. 3).

Lactobacillus casei ssp. *casei* grown in MSE broth (18 h, 30°C) exhibited a layer of polysaccharide on its surface (Fig. 4) intensively stained by ruthenium red. However, the medium did not appear viscous.

Biosynthesis and growth of new grains formed by L. hilgardii

The pieces of gel embedding *L. hilgardii* (above) increased in volume after they had been transferred to a SY solution with 0.1% (w/v) of peptone. After two sub-cultures in the latter medium, the gels acquired the same toughness as household kefir grains. Peptone was then withdrawn from the medium for the subsequent sub-cultures. The increase of the polysaccharide (wet weight) of five samples of 300 g of grains was recorded (Fig. 5) against the pH of the solution. The average percentage of weight increase and standard error was 80 ± 15% in 21 h at 30°C, and the yield of dry polysaccharide/glucose was 62.5 ± 9.2%.

With different weights of grains (Fig. 6) the percentage of weight increases were variable, but the yields/glucose remained close to 60% on average for the highest weights. The slopes of the increase lines between 3 and 7.5 h of culture were recorded (Fig. 7). During this period, the enzyme production necessary to build the

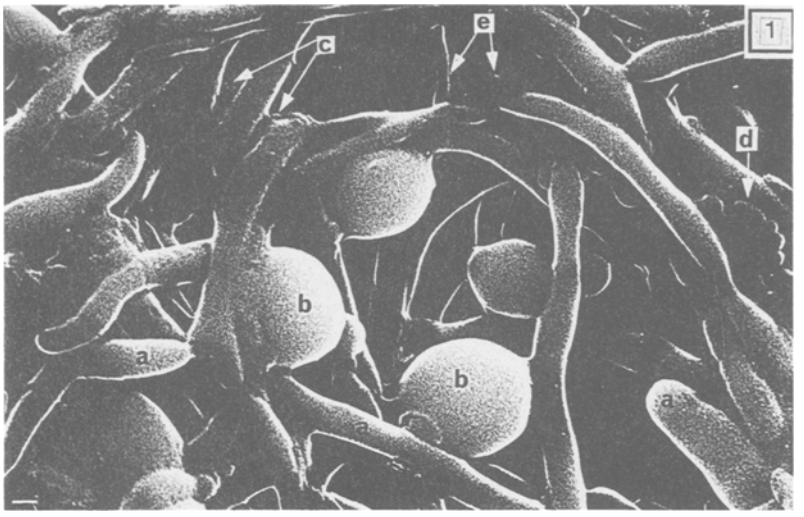

Fig. 1 Scanning electron microscopy of the outer part of a sugary kefir grain, showing a network of pseudomycelia (a) bearing blastospores (b). The filaments are stuck together by an adhesive substance (c); underneath, cocci (d) and bacilli (e) appear. (Bar = 1 μm.)

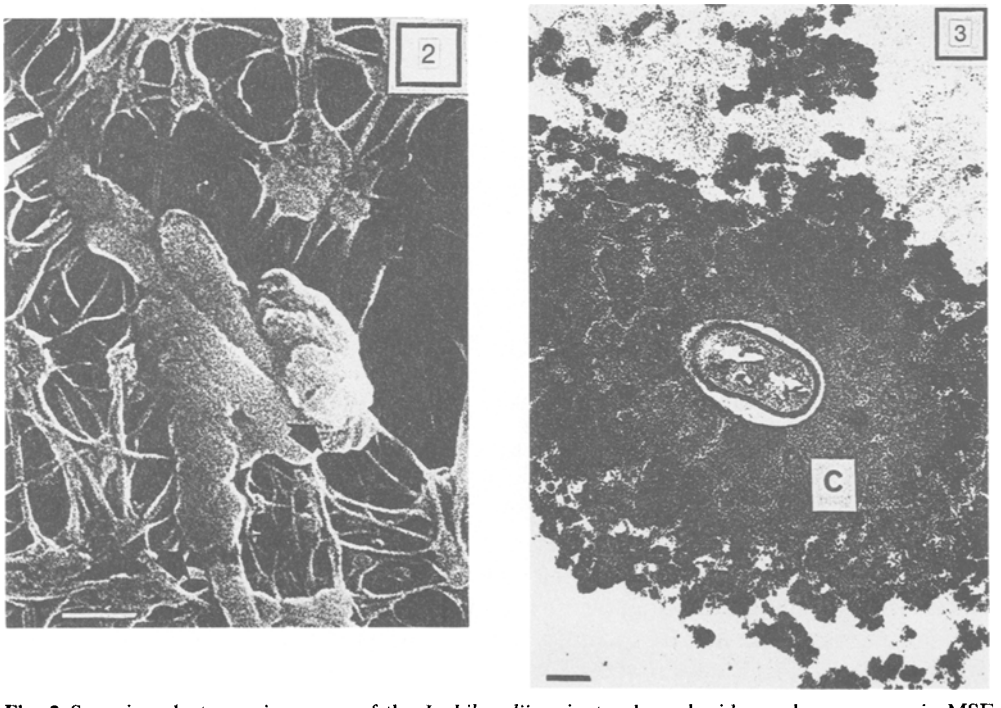

Fig. 2 Scanning electron microscopy of the *L. hilgardii* variant polysaccharide producer, grown in MSE broth (30°C, 18 h) and laid on Teflon porous membrane (0.2 μm). A deposit of insoluble substance appears on the bacteria. (Bar = 1 μm.)

Fig. 3 Transmission electron microscopy of a section of gel produced by culturing *L. hilgardii* in MSE broth (30°C, 5 days): a bacterium in transverse cross-section is surrounded by a large polysaccharidic capsule (C). Post-fixation: 1% osmic acid ruthenium red. (Bar = 0.5 μm.)

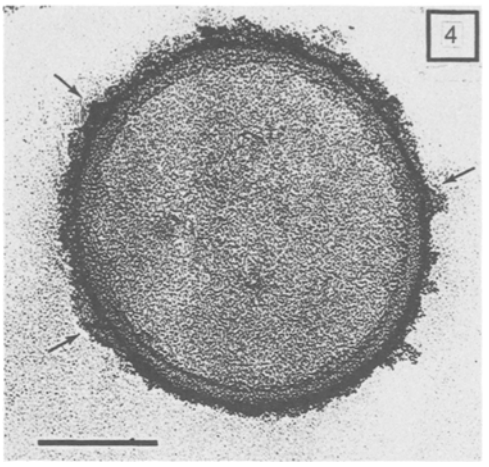

Fig. 4 *Lactobacillus casei* ssp. *casei* grown in MSE broth (30°C, 18 h) showing in transverse cross-section a polysaccharidic deposit (arrows) on the surface. Post-fixation: 1% osmic acid ruthenium red. (Bar = 0.2 μm.)

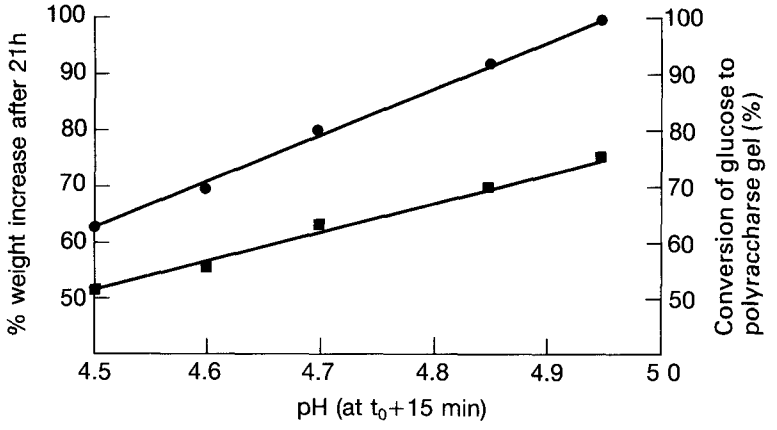

Fig. 5 Performance of *L. hilgardii* grains (five samples each 300 g wet wt) in SY solution sucrose (10% w/v), yeast extract (0.25% w/v), magnesium sulphate (0.02% w/v), pH 6.5 at 30°C in relation to pH. Weight increase after 21 h (●), percentage conversion of glucose to polysaccharide gel (■) measured 15 min after the beginning of the experiment.

polysaccharide was not yet sufficient (data to be published later). Therefore, the fact that the higher the weight of grains, the higher was the rate of increase could indicate that the exoenzyme synthesising the dextran had been produced earlier and had been retained inside the grain. Its quantity was thus proportional to the weight of gel. However, Fig. 7 also shows that the increase in productivity (g/h) was smaller as the weight of polysaccharide increased. This could be explained by the pH values of the different samples at the beginning of the culture, the highest increases being obtained with pH around 5.0 to 5.5 (Figs. 5 and 7). Because reduced pH was a result of the metabolic activity of the immobilized bacteria, pH values remained near the optimum for dextran sucrase activity for much longer when there were fewer bacteria, and the same applied to the lowest weights (Figs. 7 and 8).

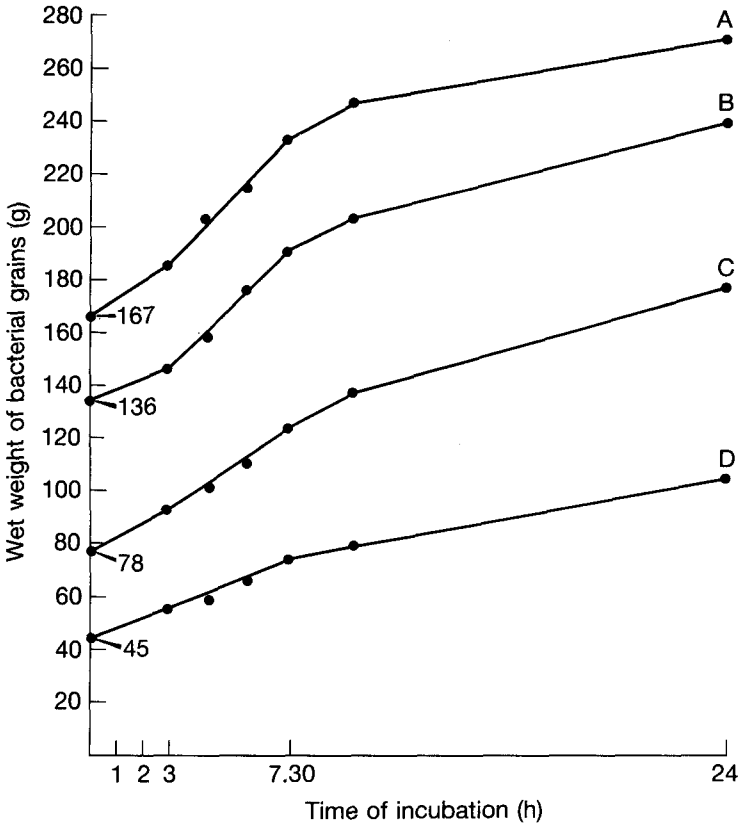

Fig. 6 Growth (wet weight) of four samples of *L. hilgardii* grains in SY solution at 30°C. Percentage conversion of glucose to polysaccharide gel at 24 h: (A) 61%; (B) 60.3%; (C) 57.5%; (D) 35%.

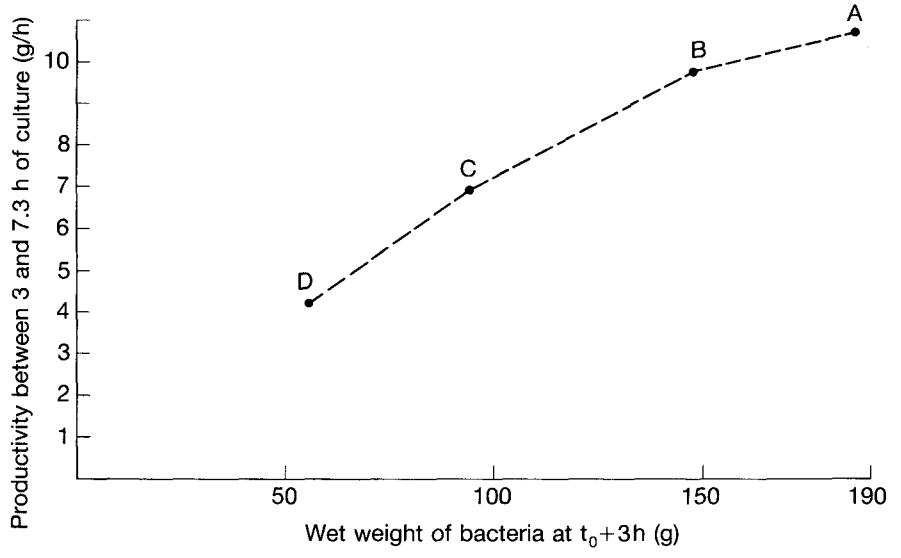

Fig. 7 Slopes of the A, B, C, D curves (see Fig. 6) between 3 and 7.30 h, expressed as productivity rates (g/h) in relation to the wet weights at 3 h (Fig. 6).

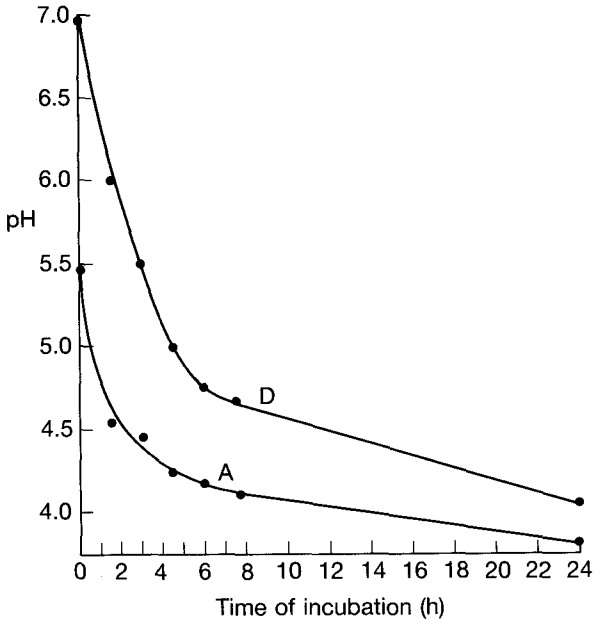

Fig. 8 pH decreases of SY solutions during the weight increase of two grains samples corresponding to the A and D curves (Fig. 6).

Further evidence of this enzymatic activity was given by the increase in polysaccharide weights of six samples of grain even at 4°C (Fig. 9). Here again, these increases were proportional to the pH values, with an average of 43.3 ± 7% in 48 h.

To improve the polysaccharide production by large quantities of grains, the pH values should be increased when the cultures were started. The simplest way was to dialyze the grains for 2 h against sterile tap water (pH 7.6, 20°C) before inoculation. The weights of two samples, 700 and 750 g, starting with pH 6.1 and 6.3, increased 57 and 62% respectively after 24 h at 30°C. These results were obtained after a dialysis of the grains confirmed that the dextransucrase is immobilized inside the grains.

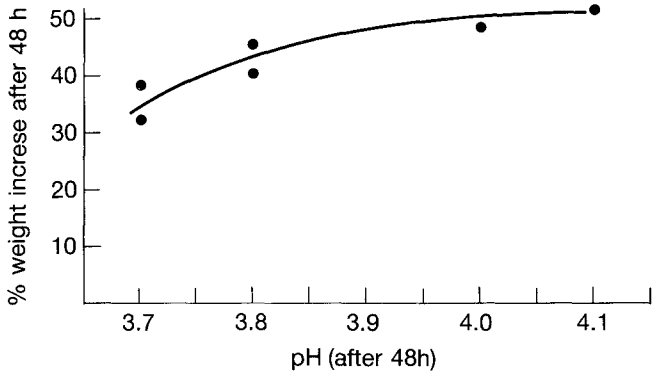

Fig. 9 Relationship between the percentages of weight increase of six samples of *L. hilgardii* grains (138 ± 13.5 g) in SY solution at 4°C, and the pH values after 48 h.

Viable counts carried out on newly synthesized grains were on average 10^9 c.f.u./g and 2×10^{10} c.f.u./g without and with dextranase treatment, respectively. A good dispersion of the bacteria after a dextranase treatment was confirmed microscopically.

When the gels were picked up from MSE agar, their general shape at that moment remained in the sub-cultures. So, the sheet-shaped grains shown in Fig. 10 derived directly from layers of gel, whereas the more rounded grains (Fig. 11) derived their shape from pieces of gel sub-cultured on MSE agar before culturing in a sucrose solution.

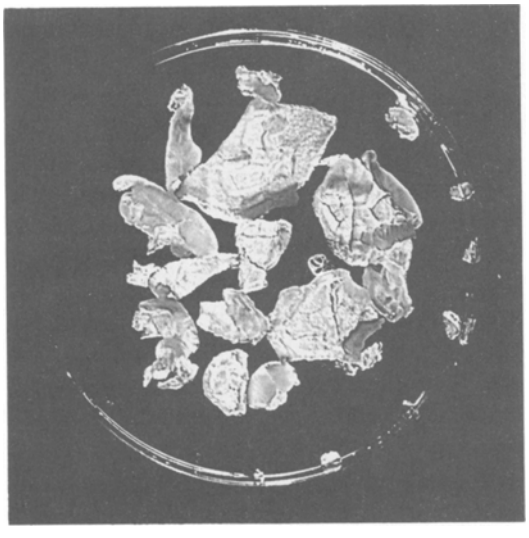

Fig. 10 New synthesized grains from *L. hilgardii* culture. The sheet forms originate from scraping pieces of gel formed on MSE agar and placing them directly in SY solution; four sub-cultures at 30°C.

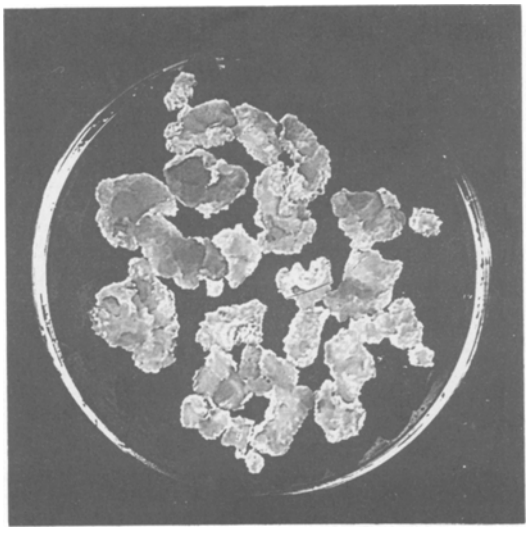

Fig. 11 As Fig. 10, but after sub-culturing pieces of gel on MSE agar.

Discussion

We have no indication of the quantitative importance of the microflora in the Tibi grains or gingerbeer plants. In sugary kefir grains, Vayssier (1978) found fewer microorganisms than in this study (1 to 2 × 10^7 compared to the present values of 2 to 3 × 10^9 c.f.u./g). This might be explained by a problem in dispersing the microorganisms which often remained fixed in particles of polysaccharide of different sizes. By comparison, Kandler & Kunath (1983), having counted 3 × 10^9 c.f.u./g in milky kefir grains, estimated that only 15% of the yeasts and 30% of the lactobacilli could be recovered on petri dishes. The partial enzymatic hydrolysis of the newly synthesized grains gave a better result than the non-treated grains (2 × 10^{10} *L. hilgardii* c.f.u./g). These counts were equivalent to those carried out on alginate or carrageenan gel beads embedded with lactobacilli. Crapisi *et al.* (1987) counted 7 × 10^{10} *L. brevis* c.f.u./g in carrageenan gel beads before the bioreactor start and 2.3 × 10^{10} after 48 h, but we have to bear in mind that our newly synthesized grains were cultured in a solution containing a small quantity of yeast extract (0.25%).

The small percentage of yeasts in household kefir grains is similar to that which occurs in the analysis of Vayssier (1978) or on milky kefir grains (Rosi & Rossi 1978). It was observed during our study that the grains degenerated if sucrose positive yeasts such as *Zygosaccharomyces florentinus* or *Torulaspora pretoriensis* were added at the rate of 10% of the total population. This was probably due to competition with *L. hilgardii* for the carbon source. These yeast strains, unable to form pseudoymycelia, were easily removed and partially eliminated in the supernatant by running fluid around the grains, thus ensuring a natural regulation of the most competitive species. Despite this competitive feature, *Z. pretoriensis* could be important in starting the fermentation of such a poor medium as sucrose solution since it is able to grow in a vitamin-free medium, as well as some *Candida valida* and *Z. florentinus* strains (Kreger van Rij 1984). *Zygosaccharomyces florentinus* was also isolated by Vayssier (1978) from kefir but was not detectable in Tibi grains (Moinas *et al.* 1980).

Since the lactic acid bacteria are well known for their essential amino acid and vitamin requirements, and our *L. hilgardii* strain required yeast extract for growing in pure culture, its association with yeasts such as those mentioned above should be a prerequisite for the grains to increase in a simple sucrose solution. We are now investigating this point. As previously reported by Wood & Hodge (1985), this association, supported by a solid polysaccharide, is typical of Tibi grains, gingerbeer plants, and milky kefir grains. 'Rather less typical is the somewhat limited nutrition afforded by the medium', reported Wood (1981) about gingerbeer plants.

The close proximity of the pseudomycelia of yeasts and lactic acid bacterial (Fig. 1) might be a key to understanding this unusual matter, but much more needs to be known about the physiological aspects of such associations.

The contaminants cited above – coliforms and faecal streptococci – appear quite frequently in household kefir grains (Vayssier 1978), resulting in off-flavours in the final product and damaging the reputation of the sugary kefir beverages. However, these contaminants did not manage to stop the growth of the grains and this resistance to a continuous infection is also a typical feature of the kefir grains (La Rivière 1967; Wood & Hodge 1985). This ability to be cultured without aseptic precautions represents another advantage of the kefir grains.

Except for *L. hilgardii*, the part played in the association by the lactic acid bacteria has not yet been defined. As an association of *L. hilgardii, L. mesenteroides* ssp. *dextranicum*, *L. casei* ssp. *casei* and yeasts (results to be published later) has been successful in producing fermented non-alcoholic beverages, we suggest that the polysaccharides possibly produced in sucrose solution by *L. mesenteroides* ssp. *dextranicum* and *L. casei* ssp. *casei* (Fig. 4) could be condensed with the dextrans of *L. hilgardii,* thus helping to retain a great number of these bacteria inside the grains.

Lactobacillus casei ssp. *casei*, forming dextran from sucrose has been found in cane juices along with *L. mesenteroides* (Tilbury 1973). The former species seems to be very adaptable with its ability to ferment a large number of different sugars. In milky kefir grains, this homofermentative species was found fixed in the grain and in small numbers in the beverage (Kandler & Kunath 1980). Some varieties of *L. casei* were also isolated from milky kefir grains from Poland and Norway which had precisely the same features of forming sediment in culture media and fermenting aldonitol (Molska *et al.* 1982).

Ward (1892) seemed to be able to form a 'plant' from a pure culture of '*Bacterium vermiforme*', adding unsterilized ginger and sugar, after 10 weeks. Daker & Stacey (1938) investigated a '*Betabacterium vermiforme*' producing polysaccharides. These invalid species names were later assigned to *L. brevis* by Horisberger (1969) but are now allocated to a close species, *L. hilgardii* (Kandler & Weiss 1986). Our *L. hilgardii* strain was previously identified as *L. brevis* (Pidoux *et al.* 1988) because of the almost identical physiological characteristics; only the DNA/DNA homology determined its allocation to *L. hilgardii*. Some slime-producing lactobacilli were studied by Sharpe *et al.* (1972), but no gel formation was reported. We attributed to our strain of *L. hilgardii* the major role in the grain formation because of its gel forming property in pure culture. Since the gel was identified as a dextran (Pidoux *et al.* 1988), the mechanism of its formation is still to be elucidated. In any case, it does not require calcium for growth and therefore the grain's structure cannot be damaged by the chelating effect of lactic acid on calcium, as happens with alginate or carrageenan gels (Boyaval *et al.* 1985).

The results presented above concerning newly synthesized grains, show the importance of a pH adjustment in order to obtain the best yields in polysaccharide production. A simple dialysis with sterile water gave good results with large quantities of grain, and like the culture itself, this phase could be operated in a continuous reactor. Since the dextrans are built by an exoenzyme such as was found for *Lactobacillus* RMW 13 (Dunican & Seeley 1963), the time for polymer production could be separated from the growing time, using different concentrations of sucrose and different pH values. This process was previously followed for the dextran production from *Leuconostoc mesenteroides* (Alsop 1983). Some optimal conditions for the polymer production will be reported later.

The shape of the newly synthesized grains is also still being investigated. The two different shapes presented (Figs. 5 and 6), corresponding to two different methods of starting with the same *L. hilgardii* strain and the same medium, can be compared with the two shapes occurring in the milky kefir grains: usually round, but sometimes a sheet-shape (Marshall *et al.* 1984).

This study has shown that the most important strain for the structure of the sugary kefir grain is *L. hilgardii* producing a polysaccharide able to gel, and the synthesis of new grains from this strain can be operated easily with satisfactory yields in an

economical way. This way of embedding and growing could be an alternative to the immobilization in alginate or carrageenan gels, with the advantage of an increase in the volume of fixed biomass. Improvements are now required to facilitate the growth of the grains and their use in a continuous reactor. The most obvious use for the grains is in the production of fermented beverages from a controlled association of lactic acid bacteria and yeasts in order to improve the organoleptic and nutritional qualities, and to maintain a low alcohol content.

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Summary

The microflora of sugary kefir grains was principally mesophilic and consisted chiefly of lactic acid bacteria [*Lactobacillus casei*, *Lactobacillus hilgardii* (= *brevis*), *Leuconostoc mesenteroides* ssp. *dextranicum*, *Streptococcus lactis*] and a small proportion of yeasts (*Zygosaccharomyces florentinus*, *Torulospora pretoriensis*, *Kloeckera apiculata*, *Candida lambica* and *C. valida*). Few coliforms and faecal streptococci were observed. Observation by scanning electron microscopy revealed that the filamentous yeasts adhered to the bacteria on the periphery of the grain.

Lactobacillus hilgardii, the single microorganism isolated which was able to produce a gelling polysaccharide, was important in the biosynthesis of the grain. Pieces of gel produced by this strain, and transferred in a yeast extract-sucrose solution, grew and resembled the household kefir grains. This represents a new, cheap way of producing immobilized cells by self-embedding in a neutral polysaccharide.

Résumé

Flore microbienne du grain de kefir sucré (plant de la bière de gingembre): biosynthèse du grain par Lactobacillus hilgardii *produisant un gel de polysaccharide*

La microflore des grains de kefir sucré est essentiellement mésophile et comprend principalement des bactéries lactiques *Lactobacillus casei, Lactobacillus hilgardii (= brevis), Leuconostoc mesenteroides* ssp. *dextranicum, Streptococcus lactis* ainsi qu'une petite proportion de levures *(Zygosaccharomyces florentinus, Torulospora pretoriensis, Kloeckera apiculata, Candida lambica* et *C. valida*). On a observé peu de coliformes et de streptocoques fécaux. L'observation au microscope électronique à balayage a révélé que les levures filamenteuses adhèrent aux bactéries sur la périphérie du grain. *Lactobacillus hilgardii*, le seul microorganisme isolé susceptible de produire un polysaccharide gélifiant, est important dans la biosynthèse du grain. Des morceaux de gel produit par cette souche et transférés dans une solution d'extrait de levure et de saccharose, croissent et ressemblent aux grains de kefir domestique. Ceci représente une manière nouvelle, peu coûteuse de produire des cellules immobilisées par auto-piégeage dans un polysaccharide neutre.