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Occurrence and Growth of Yeasts in Yogurts

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Yogurts purchased from retail outlets were examined for the presence of yeasts by being plated onto oxytetracycline malt extract agar. Of the 128 samples examined, 45% exhibited yeast counts above 10^3 cells per g. A total of 73 yeast strains were isolated and identified as belonging to the genera *Torulopsis*, *Kluyveromyces*, *Saccharomyces*, *Candida*, *Rhodotorula*, *Pichia*, *Debaryomyces*, and *Sporobolomyces*. *Torulopsis candida* and *Kluyveromyces fragilis* were the most frequently isolated species, followed by *Saccharomyces cerevisiae*, *Rhodotorula rubra*, *Kluyveromyces lactis*, and *Torulopsis versatilis*. The growth of yeasts in yogurts was related to the ability of the yeasts to grow at refrigeration temperatures, to ferment lactose and sucrose, and to hydrolyze milk casein. Most yeast isolates grew in the presence of 100 μg of sorbate and benzoate preservatives per ml. Higher yeast counts from yogurts were obtained when the yogurts were plated onto oxytetracycline malt extract agar than when they were plated onto acidified malt extract agar.

Yogurt is a fermented milk product and, traditionally, has been prepared by allowing milk to sour at 40 to 45°C. Modern yogurt production is a well-controlled process that utilizes ingredients of milk, milk powder, sugar, fruit, flavor, coloring, emulsifiers, stabilizers, and specific cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to conduct the fermentation (9, 22). In recent years, there has been a large increase in the popularity of yogurt as a food product, and this has been attributed to the use of sugar, fruits, and flavors in its manufacture (5, 14).

Because of their low pH, yogurts are a selective environment for the growth of yeasts, and the literature contains general references to the spoilage of yogurts by yeasts (8, 11, 21, 26). However, the ecology of this association has not been studied. The introduction of fruit and sugar into yogurts has amplified the risk of spoilage by yeasts by providing additional sources of contamination and fermentable substrates (5, 8). The control of this type of spoilage has become one of the main concerns of yogurt manufacturers (4, 8, 14).

The spoilage of yogurts by yeasts is generally recognized by the development of yeasty off-flavors, loss of texture quality due to gas production, and the swelling and eventual blowing off of the product container (4, 7, 14). When produced by "good manufacturing practice," yogurts should contain no greater than 1 yeast cell per g and, if correctly stored under refrigeration (5°C), a product shelf life of 3 to 4 weeks may be expected (4). However, examination of yogurts randomly purchased at retail outlets in the United Kingdom and Canada has shown that 25 to 30% of the samples contain greater than 10^3 yeast cells per g. Some samples exhibited yeast counts as high as 10^5 cells per g (1, 7, 8). The yeasts present in these yogurts were not identified.

The types of yeasts that are able to grow in yogurts and those yeast properties which enable this growth have not received detailed study. In an early study, Soulides (24) reported that lactose-fermenting yeasts of the genus *Torulopsis* were predominantly responsible for the spoilage of natural yogurts which contained no nonmilk ingredients such as sugar, fruit, and stabilizers. The introduction of sugar and fruit into yogurts makes yogurts a less selective growth environment, and such yogurts are likely to support the growth of a wider variety of yeast species. Consistent with this expectation, Tilbury et al. (25) have isolated species of *Torulopsis*, *Candida*, and *Hansenula* from fruit yogurts, but they did not report the extent to which these species had grown in the product.

This paper reports the cell numbers and species of yeasts found in some Australian yogurts and examines some properties that govern the ability of yeasts to grow in yogurts. In addition, two plating media for enumerating yeasts in yogurts are compared.

MATERIALS AND METHODS

Yogurt samples. Yogurts were purchased in sealed, 250-ml plastic containers from retail outlets in Sydney and were selected to give a cross section of

flavors and manufacturing companies. Guided by date coding, we chose for analysis only those samples that had not been manufacturered more than 5 days previously. The samples were transported in an ice box to the laboratory, where they were immediately analyzed.

Yeast enumeration, isolation, and identification. The contents of each yogurt container were uniformly mixed, and a 5.0-ml sample was aseptically withdrawn, mixed in a test tube with 5.0 ml of 0.1% sterile peptone solution, and then diluted for counting purposes. Unless indicated otherwise, all counts were made by spread-plating 0.2-ml volumes onto malt extract agar (MEA) (Oxoid Ltd.) containing 100 μg of filter-sterilized oxytetracycline (Sigma Chemical Co.) per ml. This medium (pH 5.5) is referred to hereafter as oxytetracycline malt extract agar (OMEA). In some experiments, yeasts were counted by being plated onto acidified malt extract agar (AMEA), which was MEA that had been adjusted to pH 3.5 by the addition of sterilized HCl after being autoclaved. Inoculated plates were incubated at 20 to 25°C for 4 days, and then colonies were counted.

Yeasts were isolated from OMEA plates, checked for purity by being cultured onto MEA plates, and then maintained on MEA slants. Isolates were examined for the following properties as described by Lodder (15): cellular morphology, pseudomycelium development, sporulation, carbohydrate fermentation, carbohydrate assimilation, nitrogen assimilation, growth in vitamin-free medium, starch production, cycloheximide resistance, and casein hydrolysis. Growth at 5 and 10°C was examined by inoculation of the isolates onto MEA plates and incubation at the desired temperature for 7 days. Resistance to sorbate and benzoate preservatives was determined by culturing the isolates on MEA plates that incorporated 100 μg of either potassium sorbate or sodium benzoate per ml. The sorbate or benzoate was added to the medium as a filter-sterilized solution, and the pH of the medium was adjusted to 4.0 with HCl. Plates were incubated at 20 to 25°C for 5 days and then examined for growth. Isolates were identified to species level by referring to standard keys for yeast identification (2, 15).

RESULTS

Numbers and species of yeasts in yogurts. Table 1 shows the yeast counts found in yogurts, the six most commonly isolated species, and the counts at which these species occurred. Of the 128 yogurts examined, 20% contained less than 10 yeast cells per g, and 55% contained less than 10^3 cells per g. Yeast counts in excess of 10^3 cells per g were noted in 45% of the samples. Three samples had yeast counts as high as 10^5 cells per g. A total of 73 yeast strains were isolated and identified to species level. The isolates were distributed in eight genera as follows: *Torulopsis* (25 isolates), *Kluyveromyces* (13 isolates), *Saccharomyces* (13 isolates), *Candida* (7 isolates), *Rhodotorula* (6 isolates), *Pichia* (5 isolates), *Debaryomyces* (2 isolates), and *Sporobolomyces* (2 isolates).

Torulopsis candida was the most frequently isolated species and was obtained from 21 yogurt samples. *Debaryomyces hansenii*, which is considered the perfect stage of *T. candida*, was isolated from another two samples and, in one of these, the species had grown to a level of 5×10^3 cells per g. After *T. candida*, *Kluyveromyces fragilis* (isolated from 11 samples), *Saccharomyces cerevisiae* (9 samples), *Rhodotorula rubra* (6 samples), *Kluyveromyces lactis* (2 samples), and *Torulopsis versatilis* (2 samples) were the most frequently isolated species. Table 1 shows the count ranges in which these species were found, and the data indicate that *T. candida*, *K. fragilis*, *K. lactis* and *T. versatilis* were capable of substantial growth in yogurts. There were 11 samples in which the counts of these species exceeded 5×10^3 cells per g, and there were 5 samples in which the counts had reached 5×10^4 cells per g. *S. cerevisiae* exhibited a limited tendency to grow in yogurts; its counts did not exceed 5×10^3 cells per g in any of the nine samples from which it was isolated. The oxidative pink yeast, *R. rubra*, occurred at levels of less than 100 cells per g and, on this basis, was considered as a contaminant that was unable to grow under the conditions in the yogurt. Of particular note were the single isolations of *P. toletana* and *C. krusei*, both of which had grown in yogurts to a level of 5×10^4 cells per g. Single isolations of numerous other species were obtained, but the counts in these cases were less than 10^3 cells per g.

Specific association of high counts or association of a particular yeast species with any par-

TABLE 1. *Numbers and species of yeasts in yogurts*

Count range (cells/g)	Samples in range (%)[a]	No. of times each species was isolated					
		T. candida	*K. fragilis*	*S. cerevisiae*	*R. rubra*	*K. lactis*	*T. versatilis*
$1-10^3$	55	13	7	3	6	1	
$1 \times 10^3-5 \times 10^3$	23	2	1	6			1
$5 \times 10^3-1 \times 10^4$	7	2					
10^4-10^5	15	4	3			1	1

[a] 128 yogurt samples were examined.

ticular brand or variety of yogurt was not observed. Nine of the yogurts examined were of the plain or unflavored variety, and the remainder consisted of raspberry, strawberry, blueberry, apricot, passion fruit, banana, and fruit salad varieties.

Properties of yeasts isolated from yogurts. Table 2 lists those yeast species which demonstrated a definite capacity to grow in yogurts and the ability of these species to ferment sucrose and lactose, utilize milk casein and lactic acid, grow at 5 and 10°C, and grow in the presence of 100 μg of potassium sorbate or sodium benzoate (the preservatives) per ml. Compared with other species, *T. candida* showed abundant growth on MEA after 7 days at 5°C. Since yogurts are stored at refrigeration temperatures, this is probably the main reason for the predominance of this species in the samples examined. In addition, the *T. candida* isolates were able to utilize milk casein and lactic acid and to weakly ferment sucrose. The other species listed in Table 2 did not give strong growth responses at 5°C, but they did grow at 10°C. Other properties probably accounted for the growth of these species in yogurts. For example, *K. fragilis* and *K. lactis* were strong fermenters of sucrose and lactose, and *K. fragilis* also hydrolyzed milk casein. *T. versatilis* exhibited moderate fermentations of both sucrose and lactose and also utilized milk casein. *S. cerevisiae* isolates gave a strong fermentation reaction on sucrose and were able to assimilate lactic acid. Although both *Candida krusei* and *Pichia toletana* had grown to high numbers in the yogurts from which they were isolated, they exhibited no growth at 5°C and were unable to ferment sucrose or lactose or to hydrolyze milk casein. Both species, however, were able to weakly ferment glucose and utilize lactic acid. With the exception of *P. toletana*, all the isolates grew in the presence of 100 μg of either potassium sorbate or sodium benzoate per ml.

Growth of yeasts in yogurts during storage. Fifteen containers of yogurts of assorted brands and flavors were stored at either 5 or 20°C, and at 0, 5, and 15 days, samples were removed from each container for yeast counting. The mean count of the yogurts at 0 days was 10^3 yeast cells per g. After 5 and 15 days at 5°C, this count had increased to 2×10^3 and 1×10^4 cells per g, respectively. On storage at 20°C, the respective 5- and 15-day counts were 2×10^4 and 2×10^6 cells per g. Yogurts stored at 5°C for 15 days remained acceptable on the basis of flavor, odor, and appearance. Yogurts stored at 20°C became unacceptable after 7 days. The plastic containers in which they were packaged became swollen and bloated, and the yogurt itself appeared frothy and exhibited distinct off-odors and flavors.

Methods for counting yeasts in yogurts. In three separate experimental trials, yeast counts were made from the same yogurt samples by direct plating onto both OMEA and AMEA. Overall, a total of 36 samples were compared. Table 3 shows the mean yeast counts for each trial on the two different plating media and, at the 95% confidence level, significantly higher counts were obtained on OMEA.

DISCUSSION

The majority of yogurts consumed in Australia are of the fruit variety and are prepared from ingredients of pasteurized milk, milk powder, sugar, flavoring, fruit syrup or pulp, emulsifiers, stabilizers, and coloring. Sorbate or benzoate preservatives may enter the yogurt through their permitted use as preservatives of fruit ingredients, but their final concentration in the yogurt would, in general, not exceed 100 μg/ml (19). Yogurts are generally prepared by mixing the milk, milk powder, sugar, emulsifiers, and stabilizers, heating the mixture to around 80

TABLE 2. *Properties of those yeast species that exhibited growth in yogurt*

Yeast species	Property							
	Fermentation of:		Casein hydrolysis	Utilization of lactic acid	Growth at:		Growth in:	
	Sucrose	Lactose			5°C	10°C	Sorbate (100 μg/ml)	Benzoate (100 μg/ml)
T. candida	+	−	+	+	+	+	+	+
K. fragilis	+	+	+	+	W[a]	+	+	+
S. cerevisiae	+	−	−	+	W	+	+	+
K. lactis	+	+	−	+	W	+	+	+
T. versatilis	+	+	W	+	W	+	+	+
C. krusei	−	−	−	+	−	+	+	+
P. toletana	−	−	−	+	−	+	−	−

[a] W, Weak response.

TABLE 3. *Comparison of OMEA and AMEA plating media used for the counting of yeasts in yogurts*

Experimental trial	No. of yogurt samples examined	Mean yeast count (cells/g)		$\bar{d}$[a]	SD[a]	Difference at the 95% confidence level
		OMEA	AMEA			
1	11	9×10^2	3×10^2	605	694	Yes
2	10	6×10^3	4×10^3	1,578	1,535	Yes
3	15	12×10^1	3×10^1	89	68	Yes

[a] Yeast counts were done on each yogurt sample by plating the samples onto OMEA and AMEA. The difference between the two counts for each sample was determined by subtraction, and the means of the differences ($\bar{d}$) for all the samples in each trial were calculated. The standard deviation (SD) of the differences for the samples in each trial was also calculated. These values were used in a two-tail t test for paired observations, and they indicated within 95% confidence limits whether the mean yeast counts obtained by the two plating methods were significantly different (16).

to 85°C for 15 to 30 min, cooling the mixture, and then fermenting it with pure cultures of *L. bulgaricus* and *S. thermophilus*. After fermentation, flavor, fruit, and color are added, and then the product is dispensed, packaged, cooled, and retailed (9, 22). Yeast contaminants in the first set of ingredients are destroyed by the heating temperatures and times given above, and it is generally accepted that yeast contamination of the final product arises from added fruit material or poor hygienic practices during the packaging operation (4, 5, 8, 9, 14). The association of yeasts with fruits in general is well documented (21, 26). On some occasions, we found yeasts in coloring agents, and this has prompted some manufacturers to heat pasteurize solutions of these agents before adding them to the product. To overcome the risk of yeast contamination from fruit ingredients, most yogurt producers either heat pasteurize the fruit material immediately before use or purchase the heat-processed fruit in cans from a supplier (4, 5). Since the added fruit material may constitute about 10% of the final volume of yogurt (4), it is essential that the fruit be free of viable yeasts. On a number of occasions we found levels of yeasts as high as 10^5 cells per g in heat-processed fruit pulps, suggesting inadequate heat processing. High yeast counts were particularly encountered in those fruit pulps that had been stored for some time after they were processed.

With good manufacturing practice, it is possible to obtain yogurts with a yeast count of less than 1 cell per g at the time of packaging. With proper refrigerated storage of the product, the yeast count should not exceed 10 cells per g after 3 to 7 days (4). The yogurts examined in this study were all studied within 5 days of the date of manufacture, yet only 20% showed yeast counts of less than 10 cells per g. Yeast counts in excess of 10^3 cells per g were noted in 45% of the samples, which suggested an unsatisfactory degree of contamination during production. Moreover, inadequate refrigeration after packaging and during marketing probably encouraged yeast growth and accounted for those samples with yeast counts in the range of 10^4 cells per g. This extent and level of yeast contamination are somewhat higher than those reported for yogurts in the United Kingdom (7, 8) and Canada (1).

The yeasts isolated in this study have been identified by the standard descriptions given by Lodder (15). Recent developments in the taxonomy of yeasts propose the renaming of some species previously described by Lodder. Pertinent to the present study are the proposals to rename *T. candida* as *Candida fumata* (27) and *K. fragilis* as *Kluyveromyces marxianus* (3). Barnett et al. (3) refer to *T. candida* as *Debaryomyces hansenii* and *C. krusei* as *Pichia kudriauzevii*.

The frequent isolation of *T. candida* from Australian yogurts is consistent with the studies of Tilbury et al. (25), who reported that in the United Kingdom, *T. candida*, *T. versatilis*, *Candida pelliculosa*, *Candida intermedia*, *and Hansenula anomala* were the most frequently isolated species from yogurts. However, the study did not state the cell levels at which the various species occur in yogurts. *C. intermedia* was isolated on one occasion from Australian yogurts and grew in the yogurt to a level of 2×10^3 cells per g. The association of lactose-fermenting yeasts with dairy products is well established (11, 21, 26). It was not surprising, therefore, to observe the occurrence and growth of *K. fragilis* and *K. lactis* in Australian yogurts.

As suggested by the frequent isolations of *T. candida* and *K. fragilis* (Table 1), important criteria governing the growth of yeasts in yogurts are the ability to grow well at refrigeration temperatures and to ferment sucrose, lactose, or both (Table 2). These criteria are consistent with the storage of yogurts under refrigerated conditions and the presence of sucrose and lactose as the major carbohydrates. The lactose concentration of yogurts is around 4%, and the

sucrose concentration for fruit and flavored yogurts may be between 5 and 10% (7). Davis (6) indicated that glucose and fructose may occur in yogurts through the use of invert sugar by some manufacturers and that small amounts of galactose may arise from the bacterial metabolism of milk lactose. These three sugars, therefore, could also act as fermentable substrates for yeast growth, and this could account for the growth in yogurts of species such as *P. toletana* and *C. krusei* that do not ferment lactose or sucrose.

The ability to hydrolyze milk casein might also be a significant property that governs the growth of yeasts in yogurts. The two species most frequently isolated from yogurts, *T. candida* and *K. fragilis*, gave strong casein-hydrolytic reactions. *T. versatilis* also hydrolyzed casein but to a lesser extent. Ingram (11) has previously reported the isolation of an unidentified casein-hydrolyzing yeast from yogurt. Such proteolytic species may raise the pH of yogurt through casein hydrolysis and produce bitter flavors (11, 26).

Lactic acid occurs in yogurt at concentrations of around 1% and is generated by the bacterial metabolism of milk lactose (7). Although most of the yeast species isolated from yogurts were able to utilize lactic acid as a carbon source, it is unlikely that this substrate could be assimilated under the anaerobic conditions that exist in yogurts (23). The strictly oxidative yeast *R. rubra* was a contaminant of yogurts but was unable to grow under the anaerobic conditions that existed in the product.

The use of sorbate- and benzoate-type preservatives in yogurts to restrict yeast growth and increase product shelf life has been suggested (6). Based on the data presented in Table 2, we determined that preservative concentrations greater than 100 μg/ml would be required for this purpose, since most of the yeasts isolated from yogurts grew readily at this concentration. The uses of such preservatives, however, are regulated by food laws.

The storage studies emphasize the importance of refrigeration in obtaining an acceptable shelf life for yogurts. Inadequate refrigeration leads to rapid growth of yeasts in yogurts. In this study, yogurt spoilage was evident when yeast counts had reached about 10^5 cells per g and, at 20°C, this occurred within 5 to 7 days.

Routine monitoring of yeast levels in the fruit ingredients and the final product is an important aspect of yogurt quality control. Yeasts are conveniently enumerated by direct plating onto agar media that have been acidified or contain specific antibiotics, such as oxytetracycline, to restrict bacterial growth (17, 21). Some authors have suggested most-probable-number methods for yeast enumeration (4, 13), but although these methods may offer a greater sensitivity of measurement, they are less accurate and more time-consuming. The use of acidified agar media for the enumeration of yeasts in foods has been criticized on the basis that acid-sensitive species and sublethally injured cells might not grow and that a general underestimation of the true count would result (10, 12, 20). The use of antibiotic-based media avoids these problems (17, 18). Consistent with these views, the antibiotic-based medium OMEA gave significantly higher yeast counts in yogurts than did the acidified medium AMEA.

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