Effect of various growth media upon survival during storage of freeze-dried *Enterococcus faecalis* and *Enterococcus durans*

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ABSTRACT

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Aims: The effects of three different growth media (MRS, M17 and Lee's) on survival during freeze-drying and subsequent storage of six strains of *Enterococcus faecalis* and two strains of *E. durans* were investigated.

Methods and Results: Distinct *Enterococcus* spp. strains were grown on M17, MRS and Lee's broth, freeze-dried and stored at 20°C in air under darkness. At regular intervals throughout storage, freeze-dried samples were rehydrated and then plated on M17 agar.

Conclusions: A higher survival rate during storage of dried *E. durans* was obtained when growth occurred in MRS. The same effect was not observed, however, for the majority of *E. faecalis* strains, which clearly survived better in the dried state when this organism had been grown in M17 or Lee's medium.

Significance and Impact of Study: The survival of the dried *Enterococcus* spp. tested during storage was shown to be strain-specific and dependent on the growth medium.

Keywords: *Enterococcus* spp., growth medium, preservation, viability.

INTRODUCTION

There is an increasing demand for artisanal dairy products, which is partly due to the uniqueness of such foods; in which wild strains (adventitious in raw milk) grow actively and hence contribute to the final organoleptic characteristics of such cheeses (Suzzi *et al.* 2000). Enterococci play a major role in ripening and aroma development in several cheeses. A few strains also possess probiotic properties (Klein *et al.* 1998), and in general they represent an important component of the bacterial flora of dairy products manufactured from raw milk (Morea *et al.* 1999; Durlu-Ozkaya *et al.* 2001), particularly in southern Europe (Franz *et al.* 1999). Characterization of the microflora of traditional cheeses may not only shed light on the specific mechanisms of ripening, but also contribute to their preservation via inclusion in tailor-made starter and non-starter cultures. These cultures will eventually lead to standardization of product quality and safety, without hampering it's organoleptic uniqueness. However, the industrial exploitation of lactic acid bacteria (LAB) as starter cultures (with a potential probiotic role) is dependent on preservation technologies, which are required to guarantee delivery of stable cultures in terms of viability and metabolic functions (Pamfedt and Hähn-Hagerdal 2000). Freeze-drying is often used for preservation and storage of biological samples; however, it brings about undesirable side-effects, such as denaturation of sensitive proteins and decreased viability of many cell types (Leslie *et al.* 1995).

The objectives of this work were to investigate the effects of three commonly used growth media upon the survival during freeze-drying, and subsequent storage of selected native strains of *Enterococcus faecalis* and *E. durans*.

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MATERIALS AND METHODS

Media preparation

MRS medium (LAB M, Bury, UK), initially proposed by de Man *et al.* (1960), and M17 medium (Merck, Frankfurt, Germany), initially proposed by Terzaghi and Sandine (1975), were prepared according to the manufacturer's instructions. Lee's medium was prepared from individual ingredients in our laboratory, following the original recipe by Lee *et al.* (1974). The compositions of the three media are given in Table 1.

Bacterial strains

Strains of *E. faecalis* (EF1, EF2, EF3, EF4, EF5 and EF6) and *E. durans* (ED1 and ED2) had been isolated from Portuguese hard cheeses, and characterized following Durlu-Ozkaya *et al.* (2001). The original reference cultures were maintained in cryogenic storage at −80°C on glass beads.

Culture conditions

Working cultures were maintained as slopes on M17 agar, stored at 4°C and subcultured every month. Slopes were in turn prepared from cultures grown on M17 broth and incubated at 37 °C for 24 h; M17 broth was inoculated from the M17 agar slopes and again incubated at 37 °C for 24 h. This culture was then used to inoculate, at 1% (v/v), MRS, M17 and Lee's broths, which were then incubated at 37°C for 24 h. Cells were harvested by centrifugation at 7000 ×*g* for 10 min and washed twice with sterile Ringer's solution (LAB M). Cells were suspended in sterile skim milk containing 11% (w/v) solids (LAB M), allowed to equilibrate for 1 h at room temperature (20 °C) and then frozen (−80 °C for 24 h) in plastic tubes. Each experiment was repeated three times.

Table 1 Composition of the media used for growth of *Enterococcus faecalis* and *E. durans*

Component	MRS (g l^{-1})	M17 (g l^{-1})	Lee's (g l^{-1})
Polypeptone	10	–	–
Peptone soymeal	–	5·0	–
Peptone from meat	–	2·5	–
Peptone from casein	–	2·5	–
Tryptone	–	–	10
Meat extract	10	5	–
Yeast extract	5	2·5	10
Glucose	20	–	–
Lactose	–	5·0	5·0
Sucrose	–	–	5·0
Na-β-glycerolphosphate	–	19	–
Dipotassium phosphate	2·0	–	0·5
Sodium acetate	5·0	–	–
Ammonium citrate	2·0	–	–
Tween 80	1·08	–	–
Ascorbic acid	–	0·5	–
Magnesium sulphate	0·2	0·25	–
Manganese sulphate	0·05	–	–

Freeze-drying and storage

Samples of 20 ml were desiccated under vacuum (50 mTorr for 48 h) in a freeze-drier (Martin Christ, Osterad am Harz, Germany), at room temperature (20 °C); the condenser was cooled at −55 °C. Dried cells were stored in hermetic closed containers at 20 °C in air and maintained in the darkness.

Enumeration of survivors

At regular intervals throughout storage, freeze-dried samples were rehydrated to the original volume with sterile deionized water; suitable dilutions were then plated on M17 agar by the drop count technique (Miles and Misra 1938). Three drops (20 μl each) of the suitable dilution were placed on each of three separate plates, which were examined after incubation at 37 °C for 48 h.

Statistical analysis

The mean values of three replicated viable counts, right after freeze-drying and at regular intervals during storage, were subject to analysis of variance (ANOVA) using the statistical software R (Ihaka and Gentleman 1996), at the 5% level of significance. Multiple comparison of treatment mean values, using 95% confidence intervals, was done using Tukey's honestly significant difference, and the results plotted using Trellis display so as to grasp important interactions between factors (Becker *et al.* 1996).

RESULTS

Four factors were considered with regard to viability: experimental replication, strain, growth medium and storage time. The ANOVA results encompassing these factors indicated that all main effects, some two-way interactions and the three- and four-way interactions were statistically significant (Table 2). The survival of freeze-dried *Enterococcus* spp. during storage (but not during freeze-drying) was shown to be dependent on the growth medium ($P < 0{\cdot}0001$) and on the strain ($P < 0{\cdot}0001$). The influence of the growth medium on survival during storage depended on the specific strain being tested, because the corresponding two-way interaction was significant ($P < 0{\cdot}0001$). As there were significant interactions between factors (Table 2), the effects

Table 2 ANOVA of the effects of different growth media on survival during freeze-drying and subsequent storage, of several strains of *Enterococcus faecalis* and *E. durans*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	*P*-value
Experimental replication (ER)	2	0·05	0·03	4·12	$1{\cdot}6 \times 10^{-2*}$
Strain (S)	7	30·38	4·34	664·92	$<2{\cdot}2 \times 10^{-16**}$
Growth medium (GM)	2	26·70	13·35	2045·18	$<2{\cdot}2 \times 10^{-16**}$
Storage time (ST)	8	844·89	105·61	16178·78	$<2{\cdot}2 \times 10^{-16**}$
ER × S	14	0·13	0·01	1·41	0·142
ER × GM	4	0·03	0·01	1·32	0·259
ER × ST	16	0·70	0·04	6·72	$6{\cdot}8 \times 10^{-15**}$
S × GM	14	118·34	8·45	1294·90	$<2{\cdot}2 \times 10^{-16**}$
S × ST	56	42·88	0·77	117·31	$<2{\cdot}2 \times 10^{-16**}$
GM × T	16	14·22	0·89	136.11	$<2{\cdot}2 \times 10^{-16**}$
ER × S × GM	28	0·28	0·01	1·53	$3{\cdot}7 \times 10^{-2*}$
ER × S × ST	112	0·62	0·01	0·85	0·861
ER × GM × ST	32	0·21	0·01	1·02	0·435
S × GM × ST	112	95·61	0·85	130·78	$<2{\cdot}2 \times 10^{-16**}$
ER × S × GM × ST	224	2·07	0·01	1·41	$<1{\cdot}9 \times 10^{-4**}$
Residuals	1296	8·46	0·01		

$^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}0001$.

on survival of different strains during storage could not be estimated only from the treatment mean values. Due to the complexity of such analysis (which consisted of comparing the differences between each strain for every growth medium in three replicated experiments), and as there were interactions between factors, the best way to visualize those effects was to plot all combinations using a Trellis display (Fig. 1).

All strains tested underwent decrease in viability to some extent during dehydration, and a greater (but variable) decrease during storage. *Enterococcus durans* strains ED1 and ED2 clearly survived better in the dried state when cells had been grown in MRS. By the end of the storage period, there were no significant differences between the survival of ED1 and ED2 on Lee's and M17 media. During the period tested, the survival of *E. faecalis* strain EF1 during storage was not dependent on the growth medium used; *E. faecalis* strains EF3 and EF6 exhibited significantly higher survival rates during storage in dried form when the growth medium was M17. There were no significant differences between survival on Lee's and MRS media for strain EF3; however, in two of the experiments, strain EF6 survived better in Lee's than in MRS medium. Lee's and M17 would apparently be the best media for growth of strain EF5 if the objective function were maintenance of high viability during storage. A similar conclusion was possible for the strain EF4, except that there were no significant differences in survival in Lee's and MRS media. The highest viability during storage in the dried state for strain EF2 was attained when growth was in Lee's medium.

Measurements tabulated in Table 3 indicate that the final pH was higher (by *ca.* 1·0 pH unit) when growth was in M17 than in MRS or Lee's media.

DISCUSSION

Our research effort provided evidence of the impact of the growth medium on viability throughout storage of dried *Enterococcus* spp. Various studies (Leslie *et al.* 1995; Linders *et al.* 1997a,b; Abadias *et al.* 2001) have been carried out to evaluate the efficacy of different components added to the drying medium upon survival of microorganisms during drying and subsequent storage; our results showed, in addition, that the growth medium is a critical parameter, which is more likely to play a role upon survival following freeze-drying. The mechanisms underlying the protection afforded by the various media tested are rather complex, and the results of previous studies do not allow final conclusions to be drawn on the mode of action of those media. Nevertheless, when taking into account the compositions of the three media, tentative reasonings may be proposed.

Several factors have been identified which could affect the protection provided by the various media (e.g. carbohydrate sources, manganese sulphate, Tween 80 and ascorbic acid). The presence or absence, as well as the different concentrations of undefined components (e.g. peptones, tryptone, meat and yeast extract) might fulfil some requirements and lead to accumulation of different compounds, and would thus be responsible for the distinct survival behaviours observed. For instance, meat extract and yeast extract are the main sources of carnitine and betaine, respectively, some of the most important compatible solutes found in LAB; hence, accumulation of these solutes will promote survival of bacteria subject to drying (Kets and de Bont 1994; Kets *et al.* 1996).

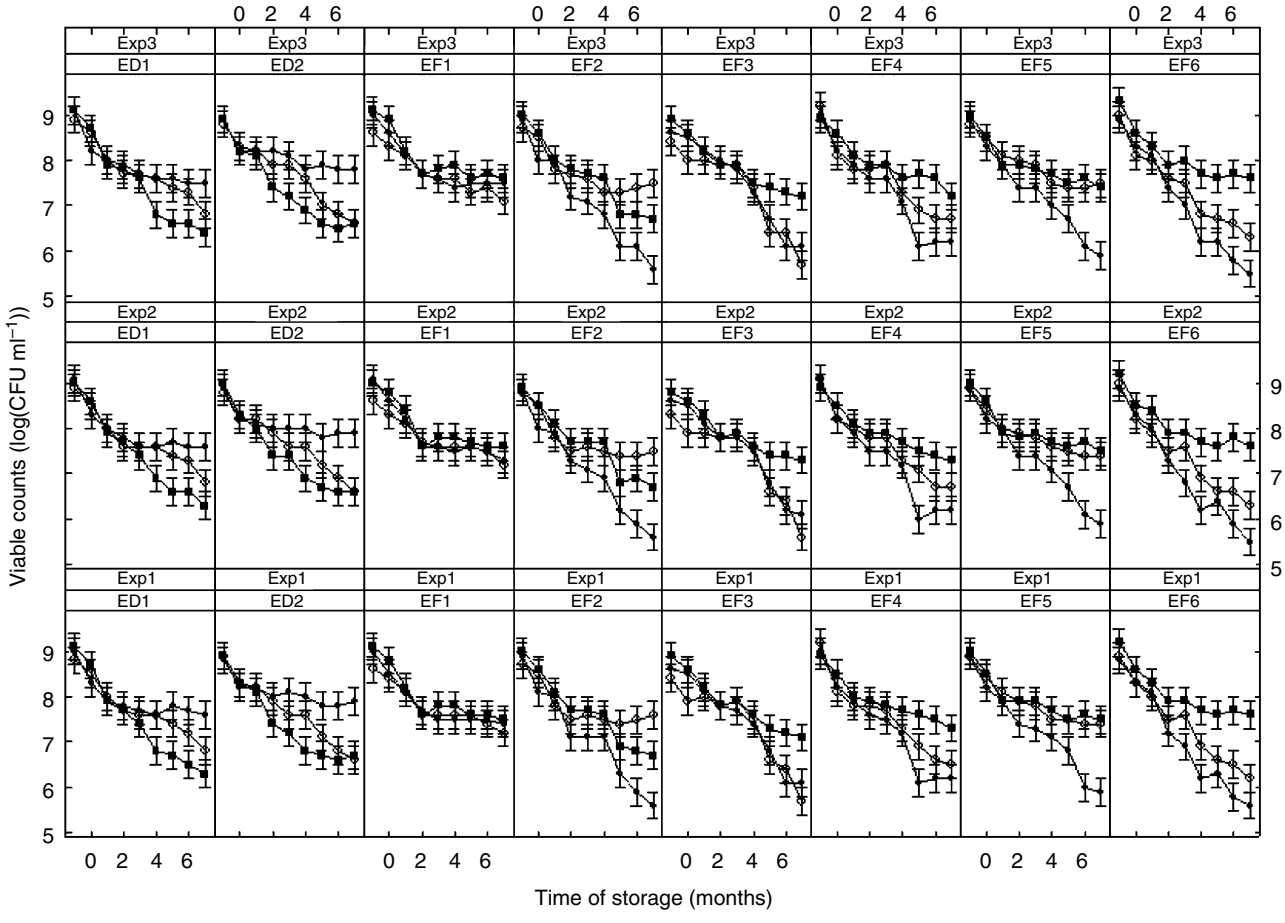

Fig. 1 Effect of various growth media: MRS (●), M17 (■) and Lee's (○) on survival during freeze-drying and subsequent storage, of several strains of *Enterococcus faecalis* and *E. durans*. Bars represent the 95% confidence intervals of treatment mean values

Table 3 Variation of pH brought about by growth of several strains of *Enterococcus faecalis* and *E. durans* in various media

	MRS		M17		Lee's	
	pH_i	pH_f	pH_i	pH_f	pH_i	pH_f
EF1	6·6	4·4	7·4	5·4	7·2	4·4
EF2	6·6	4·5	7·4	5·4	7·2	4·5
EF3	6·6	4·5	7·4	5·5	7·2	4·5
EF4	6·6	4·6	7·4	5·5	7·2	4·4
EF5	6·6	4·7	7·4	5·5	7·2	4·7
EF6	6·6	4·6	7·4	5·5	7·2	4·4
ED1	6·6	4·4	7·4	5·5	7·2	4·2
ED2	6·6	4·4	7·4	5·5	7·2	4·3

pH_i – initial pH of the medium.
pH_f – final pH of the medium, after strain growth at 37°C for 24 h.

In our studies, the strains of *E. faecalis* and *E. durans* were exposed to different components of the growth media, e.g. presence or absence of Tween 80 and ascorbic acid, which may yield different fatty acid profiles and different degrees of oxidation of membrane lipids in those microorganisms; such fatty acid composition and degree of oxidation seems to be related to survival of cells during freeze drying (Goldberg and Eschar 1977; Murga *et al.* 2000) and subsequent storage

in the dried state (Castro *et al.* 1996; Teixeira *et al.* 1996). It has previously been described (Smittle *et al.* 1974; Goldberg and Eschar 1977; Kimoto *et al.* 2002) that culturing with Tween 80 changes the fatty acid composition of LAB cells, and that this change influences their subsequent resistance to other forms of stress. However, our studies are in agreement with Kimoto *et al.* (2002) who claimed that such modification on the fatty acid profile is strain-dependent. The addition of ascorbic acid to the drying medium had already been demonstrated to have a protective effect on spray-dried cell concentrates of *Lactobacillus bulgaricus* during storage (Teixeira *et al.* 1995).

Another possible explanation for the differences observed relates to the different carbon sources in the three media. Studies by Hofvendahl and Hahn-Hägerdal (2000) showed that other compounds are formed by homofermenters in addition to lactic acid, during growth on sugars other than glucose. It is therefore possible that the different chemicals produced during fermentation of sugars might be responsible for the distinct survival behaviours during storage of freeze-dried *E. faecalis* and *E. durans*. This hypothesis is corroborated by the different final pH values achieved in the media tested.

Our findings also back up an alternative explanation. Acid shock or adaptation thereto may yield cells that exhibit alternative physiological states, and hence potentially different tolerances to other stresses (Abee and Wouters 1999). Previous studies (Giard *et al.* 2000) have indeed shown that starving *E. faecalis*, caused by glucose exhaustion, results in development of a multiresistant state that protects the starved cells from other stresses. However, Rince *et al.* (2000) reported that adaptation of *E. faecalis* to acidic pH resulted in only a small effect by other challenges. In our studies, adaptation to low pH seems to produce cells with different sensitivities to further stress (e.g. storage in the dried state). Owing to such a heterogeneous behaviour, no conclusions can be drawn pertaining to the relationship between pH adaptation and subsequent resistance in the dried state. It was also reported (Tourdot-Maréchal *et al.* 2000) that adaptation of *Oenococcus oeni* to acidic conditions had no effect on membrane fluidity, in contrast to other types of adaptations.

Of the three media tested in this study, only MRS contained manganese. Note that for most LAB deprived of Mn (II), sensitivity to oxygen is increased as (i) it may substitute superoxide dismutase (SOD) or (ii) it is essential for SOD activity, hence protecting the cell against the direct and indirect toxic effects of reactive oxygen species (Archibald and Fridovich 1981); such species have numerous cellular targets, e.g. DNA and membrane, thus leading to strand breaks and peroxidation of lipids, respectively (Flahaut *et al.* 1998). A manganese-dependent SOD was identified in various enterococcal strains (Poyart *et al.* 2000) and the gene encoding for Mn-dependent SOD was different for the two enterococcal species used in these studies.

Our data indicate that distinct strains can differ in their behaviour during storage in the dried state, but the reasoning behind that realization is unclear. It was suggested (O'Callaghan and Condon 2000) that gene transfer between strains may have led to differences in phenotype between distinct *Lactococcus lactis* strains. Although cause and effect mechanisms require further investigation, our results already point to the importance of the growth medium on the survival during storage of freeze-dried enterococcal strains. It is also important to realize that, for each LAB strain of interest, the influence(s) of growth conditions, and probably the composition of the drying medium on the survival in the dried state, are specific for that strain.

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