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Hosts, environment, stress, phages

Application of *Streptococcus thermophilus* DPC1842 as an adjunct to counteract bacteriophage disruption in a predominantly lactococcal Cheddar cheese starter: use in bulk starter culture systems

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Abstract — A significant amount of Cheddar cheese manufactured world-wide relies on bulk starter cultures instead of direct vat set (DVS) cultures. While the inclusion of *S. thermophilus* is sometimes used to counteract failure due to lactococcal phage in the latter system, it is considered difficult to implement in bulk starter systems and is normally avoided. This stems from the problem in controlling the ratio of *S. thermophilus* to lactococci during the bulk starter preparation such that suitable acidification rates can be achieved. The current study demonstrates how *S. thermophilus* numbers can be controlled during growth in the bulk starter medium prior to inoculation of a culture, based on three lactococcal strains and *S. thermophilus* DPC1842, into the cheese vat. The concentration of inorganic phosphate necessary to inhibit the growth of strain DPC1842 in a whey-based bulk starter medium was found to be 0.18 mol·L^{-1}. Since higher levels of phosphate exist in different commercial bulk starter media used for mesophilic cultures, a number of these media can be used for propagation of this starter blend without domination of strain DPC1842 over its lactococcal counterparts. Strain DPC1842 is highly phage resistant and is particularly acid-fast in the cheese milk and reduces the pH efficiently at very low inocula. Cheddar cheese was manufactured in a commercial plant with this system and the resulting cheese had good flavour characteristics. This study demonstrates the effectiveness of the *S. thermophilus* DPC1842 component in rescuing the fermentation in the event of severe lactococcal phage attack.

***Streptococcus thermophilus* / adjunct / Cheddar / bacteriophage**

Résumé — Ajout de *Streptococcus thermophilus* DPC1842 à la flore lactococcale utilisée en fabrication de Cheddar pour contrecarrer les attaques phagiques : utilisation dans des systèmes de culture de levain industriel. Une partie importante du fromage de Cheddar fabriqué dans

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le monde repose sur l'utilisation de cultures de levains industriels au lieu de cultures d'ensemencement direct en cuve. Alors que l'ajout de *S. thermophilus* est parfois utilisé dans ce dernier système pour contrecarrer la défaillance due aux phages de lactocoques, ceci est considéré comme difficile à mettre en œuvre dans les systèmes de levains industriels et est normalement évité en raison de la difficulté à contrôler, au cours de la préparation du levain industriel, le rapport *S. thermophilus*/lactocoques et donc à obtenir un taux d'acidification convenable. La présente étude montre comment le nombre de *S. thermophilus* peut être contrôlé pendant la croissance dans le milieu de culture du levain industriel avant l'inoculation dans la cuve de fromagerie d'une culture composée de 3 souches de lactocoques et de la souche de *S. thermophilus* DPC1842. La concentration de phosphate inorganique nécessaire pour inhiber la croissance de la souche DPC1842 dans le milieu de culture du levain industriel à base de lactosérum a était de 0,18 mol·L^{-1}. Étant donné que différents milieux du commerce, utilisés pour des cultures mésophiles de levains industriels, renferment des concentrations supérieures de phosphates, un certain nombre de ces milieux peut être utilisé pour cultiver ce mélange de levain sans domination de la souche DPC1842 sur les lactocoques. La souche DPC1842 est très résistante aux phages, acidifie très rapidement le lait de fromagerie, et réduit le pH efficacement à des taux d'inoculation très bas. Du Cheddar, fabriqué commercialement selon cette technologie, présentait d'excellentes qualités aromatiques. Cette étude démontre l'efficacité de la souche de *S. thermophilus* DPC1842 pour restaurer la fermentation en cas d'attaque sévère par les phages de lactocoques.

***Streptococcus thermophilus* / Cheddar / bactériophage**

1. INTRODUCTION

Cheddar cheese starters are nowadays generally composed of 2–6 well-characterised *Lactococcus lactis* strains which are chosen for their fast acid-producing ability and their high phage insensitivity [11] and their ability to perform within stringent manufacturing schedules. Currently in the cheese industry, manufacturers are relying to an increasing extent on small numbers of lactococcal strains for a reliable process and a consistent high quality end product. The destructive potential of phage is exaggerated in modern processes which employ cultures on a continuous basis and where huge numbers of starter cells are required to process large volumes of milk to cheese. Despite the fact that cheese-making strains are initially chosen on the basis of phage resistance, they frequently become susceptible to phages, which emerge after extended use in the cheese plant [1, 6, 8, 13]. Important sources of these phages include the external environment and evolution from pre-existing phages within the cheese plants themselves [4, 6]. The use of *S. thermophilus* strains as phage-unrelated starter components with lactococci in direct-vat-set (DVS) cheese cultures for use in mesophilic fermentations is common [7]. The reasoning behind this is that *S. thermophilus* will continue to produce acid in the cheese vat in the event of phage associated disturbances of the lactococcal starter components. Since a significant amount of Cheddar cheese production relies on bulk starter cultures instead of DVS cultures, the inclusion of *S. thermophilus* can be difficult and is normally avoided. This stems from the problem in controlling the ratio of *S. thermophilus* to lactococci during the bulk starter preparation such that suitable acidification rates can be achieved in the cheese vat. This study demonstrates an effective control of the ratio of *S. thermophilus* to lactococci from the time of inoculation of bulk starter (with a blend of streptococci and lactococci) to cheese manufacture. It also shows the effectiveness of the *S. thermophilus* DPC1842 component in completing the fermentation in the event of severe lactococcal phage attack where in excess of 99.9% of the *Lactococcus* population was eliminated.

2. MATERIALS AND METHODS

2.1. Bacterial strains, bacteriophage and media used

The bacteria and bacteriophage used in this study were obtained from the culture collection at the Dairy Products Research Centre. Strains of *L. lactis* were propagated in either 10% (w/v) reconstituted skim milk (RSM), or M17 medium (Difco Laboratories, Detroit, USA) at 30 °C and supplemented with 0.5% (w/v) lactose (LM17). Strains of *S. thermophilus* were propagated in LM17 medium or RSM at 42 °C. Commercially available bulk starter medium was heated to 90 °C for 30 min prior to use. To aid in differentiating between lactococci and streptococci in mixed starter populations, X-Gal (5-Bromo-4-chloro-3-indolyl-β-galactoside) was incorporated into LM17 at a final concentration of 0.02%. *L. lactis* produced white colonies and *S. thermophilus* produced small blue colonies when grown on LM17 containing X-Gal. Non-starter lactic acid bacteria (NSLABs) from cheese were enumerated on LBS agar as described previously [16].

2.2. Characterisation of acid-production by starter strains

Cheddar cheese temperature profiles (CTP) were employed to assess acid producing ability of starter strains in milk as described previously [5].

2.3. Laboratory scale cheese manufacture

Laboratory scale cheese trials were performed using 2 L of pasteurised whole milk, which was heated to 32 °C prior to inoculation. A 1.5% inoculum was added and stirred for 35 min until a pH of 6.6 had been achieved. Chymax-Plus rennet (Chr. Hansen Laboratories, Little Island, Cork, Ireland) at a concentration of 0.18 ml·L^{-1} was then added. The milk was allowed to set for approximately 40 min after which the curd was cut using a sharp knife. The curd was then cooked at 38 °C. Whey was drained when curd reached a pH of 6.1. pH of curd was monitored periodically until it reached 5.2 when it was placed in small plastic cylindrical cheese moulds with draining pores of 3 mm in diameter (Cat. No. 3404 m, Smallholding supplies, Pikes farmhouse, Somerset, UK). Curd was pressed overnight at 4 °C and ripened as required.

2.4. Measurement of intracellular enzyme release in cheese curd

Following cheese manufacture including overnight pressing, triplicate samples of cheese curd were placed in 10 mL tubes and centrifuged at 50 000 rpm for 30 min at 4 °C in a Sorvall® OTD65B ultracentrifuge. Curd juice was removed from the tube and assayed for lactate dehydrogenase (LDH), an intracellular marker enzyme indicating cell lysis. This was performed using the method of Wittenberger and Angelo [18], which measures the decrease in absorbance at 340 nm resulting from the pyruvate dependant oxidation of NADH. LDH activity was expressed as activity units (AU) per mL of juice, where 1 unit is the amount of enzyme that catalyses the oxidation of 1 μmole of NADH per min per mL. The LDH levels presented are the averages of those obtained from triplicate analyses of cheese curds.

2.5. Pilot-scale Cheddar cheese manufacture

Pilot-scale cheese making trials and cheese analyses were performed as described previously [16].

2.6. Industrial-scale Cheddar cheese manufacture

Industrial-scale Cheddar cheese making trials and analyses were performed as

described previously [10]. The mesophilic bulk starter medium employed by the cheese plant was PIM100 (Nutrition Supplies and Services Ltd., Inishannon, Co. Cork, Ireland).

3. RESULTS AND DISCUSSION

3.1. Starter culture design

A typical starter system for Cheddar manufacture is based on 1 or 2 acid-fast phage resistant lactococcal strains and 1 or 2 autolytic lactococcal strains which enhance starter enzyme release into the cheese. The system used in this study included 2 acid-fast phage resistant lactococci, 1 autolytic *Lactococcus* strain with the additional component, *S. thermophilus* to ensure good acid production in the event of severe lactococcal starter inhibition due to phage. The *S. thermophilus* strains selected were not affected by bacteriophage cocktails which were inhibitory to lactococcal starters nor were they affected by existing cocktails of thermophilic phage from 2 Irish Mozzarella cheese plants.

3.2. Performance of *S. thermophilus* during Cheddar CTP

A number of strains of *S. thermophilus* were used as adjuncts with commercial Cheddar cheese starters in pilot scale cheese manufacture. Three strains were identified which demonstrated resistance to phages from Mozzarella plants and appeared to have no influence on Cheddar cheese flavour (data not shown). These 3 strains of *S. thermophilus* were compared for their acid producing ability in milk over the CTP. Of these, strain DPC1842 was particularly acid-fast (Fig. 1). Over the Cheddar CTP, an inoculum level of 0.025% of this strain was sufficient to reduce the pH of 10% RSM to 5.2 within 6 h. The ability to acidify milk efficiently at such a low inoculum has obvious advantages in situations where lactococcal components

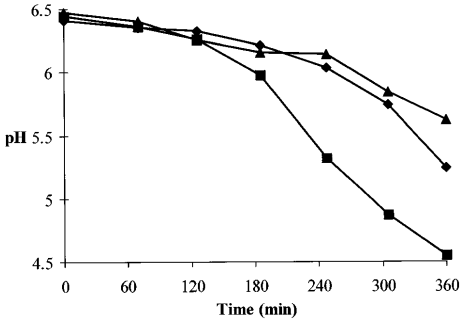

Figure 1. pH development in 10% RSM incubated over the Cheddar cheese temperature profile with a 1.5% inoculum of *S. thermophilus* DPC1854 (◆); *S. thermophilus* DPC1150 (▲); and *S. thermophilus* DPC1842 (■).

of a starter are inhibited by phage as discussed later in this communication. Importantly, this ability would suggest that the strain might have a high probability of dominating the starter population when combined and grown with lactococci in the bulk starter tank and cheese vat.

3.3. Formulation of different starter blends

Seven different four-strain blends were assembled. These were formulated as *S. thermophilus* DPC1842 with two acid-fast phage resistant lactococci and one autolytic lactococcal strain at a ratio of 1:1:1:2, respectively. Rates of acid production of all blends in milk incubated over the Cheddar cheese temperature profile were monitored. Two blends were chosen for pilot-scale Cheddar manufacture due to their superior acid-producing capabilities. These were blend 1: *S. thermophilus* DPC1842/*L. lactis* DPC4830/*L. lactis* DPC4987/*L. lactis* DPC4991 (autolytic) and blend 2: *S. thermophilus* DPC1842/*L. lactis* DPC5020/*L. lactis* DPC4988/*L. lactis* DPC4990 (autolytic) which could reduce the pH of milk to 4.96 and 4.75,

respectively. Following pilot-scale cheese manufacture, cheeses were assessed for composition, body and flavour. Both cheeses graded well and compared favourably with control cheese manufactured with a commercial Cheddar cheese starter, namely 303/227 (Chr. Hansen Laboratories, Little Island, Cork, Ireland). However, the cheese made from blend 1 was considered superior to that of blend 2 on the basis of assessment by commercial cheese graders and thus blend 1 was used for the remainder of the study.

3.4. Population dynamics of streptococci and lactococci in bulk starter media

Commercial Cheddar cheese manufacture with bulk starter in Ireland is generally based on propagation of the starter culture in whey-based medium overnight followed by activity tests on the fully-grown bulk starter culture the following day. Typically in the Irish industry, the culture is grown in the bulk starter medium for 18 h at 25 to 27 °C prior to inoculation into cheese vats. For this reason, it was important to study the population dynamics of *L. lactis* and *S. thermophilus* from the time of inoculation in the bulk starter medium through propagation and inoculation into the cheese vat and throughout cheese manufacture.

The behaviour of *S. thermophilus* in relation to lactococci was first determined in bulk starter medium (PIM100) by performing differential plate counts after inoculation (T = 0) and after incubation at 27 °C for 18 h (T = 18). For comparison sake, the strain *L. lactis* DPC4268 alone was used as the lactococcal component. The ratios tested included *Lactococcus: Streptococcus* at 1:0, 0:1, 1:1, and 3:1. In all cases where lactococci were present, they grew very well reaching approximately 10^{10} cfu·mL^{-1} of bulk starter medium at T = 18. In contrast, with *S. thermophilus*, the counts at T = 18 were never greater than the count at T = 0 indicating that *S. thermophilus* DPC1842 did not grow in the bulk starter medium under these conditions even when no lactococci were present. The same result was observed in another commonly used mesophilic bulk starter medium namely Economie 6 (Rhodia Texel, Ltd., UK).

3.5. Inorganic phosphate in bulk starter medium inhibits growth of *S. thermophilus* DPC1842

Commercial bulk starter media are generally prepared from deionised whey supplemented with protein hydrolysates, ammonium and sodium phosphate salts [2, 17]. Mesophilic bulk starter media also contain high levels of inorganic phosphates [3, 9, 15], which have been found to inhibit the activity of β-galactosidase in *S. thermophilus* [14]. The growth of strain DPC1842 was analysed in a model starter medium based on demineralised whey (10%), yeast extract (0.5%) and ascorbic acid (0.05%) [15]. This was supplemented with Na_2HPO_4 at a variety of concentrations ranging from 0.01 mol·L^{-1} to 0.25 mol·L^{-1}. It was observed, after incubation at 27 °C for 18 h, that 0.18 mol·L^{-1} Na_2HPO_4 was the threshold level at which the number of *S. thermophilus* DPC1842 at T = 18 did not exceed the number at T = 0 (8.5×10^6 cfu·mL^{-1}) (Fig. 2a). In the absence of Na_2HPO_4, DPC1842 reached 1.1×10^9 cfu·mL^{-1}. Lactococcal growth, on the other hand, generated a final count of 7.9×10^9 cfu·mL^{-1} in the presence of 0.18 mol·L^{-1} Na_2HPO_4 (Fig. 2b). These trends were also evident in both of the commercial mesophilic bulk starter media examined. From these observations, we conclude that *S. thermophilus* DPC1842 can be present at a similar level to any of the *L. lactis* components at the time of inoculation into the bulk starter medium without any risk of out-competing the lactococci.

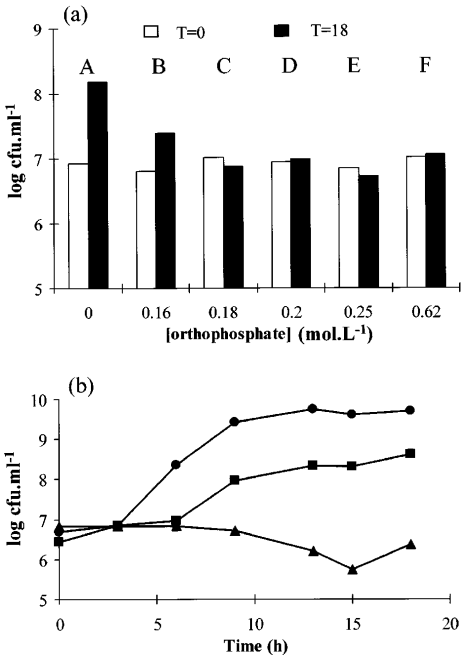

Figure 2. (**a**) Inhibitory effect of increasing orthophosphate concentration on the growth of *S. thermophilus* DPC1842 in a whey-based medium supplemented with (A) no phosphate; (B) 0.16 mol·L^{-1} orthophosphate; (C) 0.18 mol·L^{-1} orthophosphate; (D) 0.2 mol·L^{-1} orthophosphate; (E) 0.25 mol·L^{-1} orthophosphate compared with (F) Commercial bulk starter medium. Bacterial counts were taken at time zero (white) and after 18 h (black). (**b**) Growth characteristics of *S. thermophilus* DPC1842 in medium A above (■) (no Na_2HPO_4) and C above (▲) (0.18 mol·L^{-1} Na_2HPO_4) and *L. lactis* DPC4932 in medium C (●).

3.6. Challenge of a typical lactococcal component of the starter with lytic phage during growth in cheese milk

A laboratory-scale model of the starter blend was made combining *S. thermophilus* DPC1842 with a commonly used lactococcal starter for which a highly virulent prolate-headed phage was available (namely phage 4932). *L. lactis* DPC4932 and *S. thermophilus* DPC1842 were grown (1:1) in commercial bulk starter medium for 18 h at 27 °C. This culture was used to inoculate pasteurised milk which was subsequently shown to contain 9×10^7 cfu *L. lactis* per mL of milk at time zero (Fig. 3). The sample was incubated over the CTP. Acid development and lactococcal versus streptococcal counts were monitored. In samples where ϕ4932 (approximately 10^5 plaque forming units.mL^{-1} cheese milk at time zero) was included, the pH still reached 5.2 when DPC1842 was present, even though counts of lactococci went as low as 2.4×10^5 cfu·mL^{-1} in the cheese milk due to the presence of disturbing phage. In this case, the time required to reach pH 5.2 was on average 60 min longer than when phage were excluded. It is, however, noteworthy that this is an extreme case in which more than 99.9% of the lactococci had been eliminated by phage. Even in this instance, which could be viewed as the worst possible case scenario, *S. thermophilus* DPC1842 did reduce the pH sufficiently in the cheese milk.

3.7. Commercial cheese manufacture

Two commercial trials were performed at an industrial cheese plant with the *Lactococcus/Streptococcus* blend 1. Cheeses were assessed on the basis of composition and flavour at 6 months. Cheese compositional analysis after 6 months ripening indicated that the test cheese had a pH of 5.17, a salt-in-moisture (S/M) of 5.50, a protein content of 25.72% and moisture in non-fat substance (MNFS) was 55.50%. These values were within parameters expected for Cheddar cheese [12]. Commercial cheese graders assessed cheese flavour, body and texture and the test compared well with premium Cheddar manufactured with commercial starter cultures on the same day. Changes in populations of the *L. lactis* and *S. thermophilus* components and NSLABs during ripening of the Cheddar made with

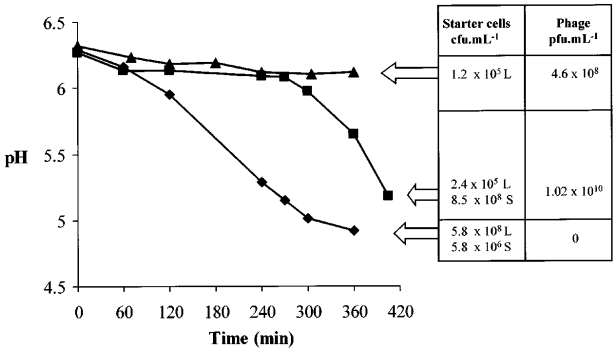

Figure 3. pH development over the CTP in 10% RSM containing *S. thermophilus* DPC1842 and *L. lactis* DPC4932 (◆); *S. thermophilus* DPC1842, *L. lactis* DPC4932 and lytic phage 4932 (■); *L. lactis* DPC4932 and phage 4932 (▲). Cell numbers at the end of the CTP are shown to the right (L = *L. lactis* and S = *S. thermophilus*). Phage titres at the end of the CTP are shown on the far right.

blend 1 are shown in Figure 4. Numbers of *S. thermophilus* gradually reduced in parallel with the reduction in numbers of *L. lactis* over the six-month ripening period. While numbers of *S. thermophilus* were at all times lower than those of *L. lactis*, the population dynamics of *L. lactis* and NSLAB behaved as expected [16].

3.8. Starter autolysis in cheese curd

The effect of including the autolytic strain DPC4991 in the starter blend on intracellular enzyme release was demonstrated by analysing one-day-old cheese curds which had been manufactured with blend 1 and a variation of this blend which was lacking strain DPC4991. There was a significant difference in LDH release between the two curds. When DPC4991 was present an LDH level of 0.12 AU·mL^{-1} was obtained from cheese juice which was significantly higher than when DPC4991 was absent (0.07 AU·mL^{-1}). This would indicate the role of DPC4991 in enhanced starter cell lysis and concomitant release of intracellular lactococcal enzymes, which are considered to have a positive impact on Cheddar cheese ripening and flavour.

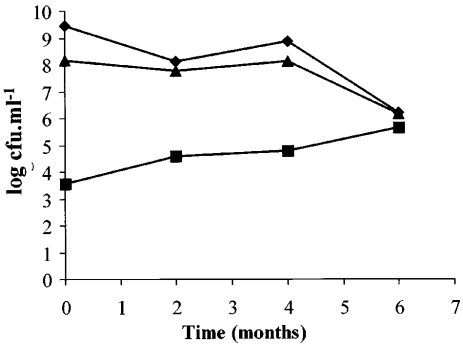

Figure 4. Survival of starter (*S. thermophilus* (▲); *L. lactis* (◆)) and NSLABs (■) in ripening Cheddar from commercial trial, made with *S. thermophilus* DPC1842; *L. lactis* DPC4830; *L. lactis* DPC4987; *L. lactis* DPC4991.

4. CONCLUSION

A number of approaches have been employed to combat the ever-present phage threat in the cheese industry [1, 6, 8, 13]. The strategy used in this study will result in sufficient acid development in cheese milk in the event of severe phage inhibition of *L. lactis* strains. The starter blend described is adapted for propagation as a bulk starter

culture. Under the conditions encountered during bulk culture preparation and Cheddar cheese manufacture, it was shown that the phosphate levels present in mesophilic bulk starter media prevented *S. thermophilus* from overtaking the lactococci unless phage inhibition occurred. Thus *S. thermophilus* DPC1842 demonstrated the capacity to rescue the Cheddar fermentation during severe phage attack. These observations may be important for both the design of bulk starter media for culture propagation and for the application of *S. thermophilus* strains as adjuncts in bulk starter cultures.

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