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# Development of a rapid, one-step screening method for the isolation of presumptive proteolytic enterococci

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ABSTRACT

Enterococci show higher proteolytic activities than other lactic acid bacteria and thus have received considerable attention in scientific literature in recent years. Proteolytic enzymes of enterococci have warranted the use of some species as starter, adjuncts or protective cultures and as probiotics, while in some strains they have also been linked with virulence. Consequently, the isolation and identification of proteolytic enterococci is becoming of increasing interest and importance. However, current screening methods for proteolytic enterococci can be time consuming, requiring a two-step procedure which may take up to 96 h. This study describes a method, utilising Kanamycin Skim Milk Aesculin Azide (KSMEA) agar, for the isolation of proteolytic enterococci in one-step, thereby significantly reducing screening time. KSMEA combines the selective properties of Kanamycin Aesculin Azide Agar (KAA) with skim milk powder for the detection of proteolytic enterococci. Enterococci produced colonies with a black halo on KSMEA which were accompanied by a zone of clearing in the media when enterococci were proteolytic. KSMEA medium retained the selectivity of KAA, while proteolytic enterococci were easily distinguished from non-proteolytic enterococci when two known strains were propagated on KSMEA. KSMEA also proved effective at isolating and detecting enterococci in raw milk, faeces and soil. Isolates recovered from the screen were confirmed as enterococci using genus-specific primers. Proteolytic enterococci were present in the raw milk sample only and were easily distinguishable from non-proteolytic enterococci and other microorganisms. Therefore, KSMEA provides a rapid, one-step screening method for the isolation of presumptive proteolytic enterococci.



## 1. Introduction

Enterococci are ubiquitous lactic acid bacteria (LAB) that constitute an important part of environmental, food and clinical microbiology. The possession of virulence traits and an increasing incidence of antibiotic resistance has culminated in the consideration of some species as opportunistic pathogens. These traits are particularly attributed to *E. faecalis* and *E. faecium* species of clinical origin (Abriouel et al., 2008; Bhardwaj et al., 2008; Kayser, 2003). However, enterococci have also been used as starter or adjuncts cultures whereby they improve the organoleptic properties of food while also proven to release natural antimicrobials, known as enterocins, shown to inhibit the growth of foodborne pathogens (Achemchem et al., 2006; Aymerich et al., 2000; Foulquié Moreno et al., 2006; Franz et al., 1999).

Certain enterococcal species have also successfully been applied as probiotics to improve human and animal health. However, enterococcal probiotics are usually not incorporated as starter or adjunct cultures, but are rather used as food supplements in the form of pharmaceutical preparations (Franz et al., 2011). Perhaps the best known and best researched probiotic *Enterococcus* strain is *E. faecium* SF68 (commercialised as Cernivet® and Cylactin®, strain NCIMB 10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland). Therefore, enterococci can be ingested in high numbers to provide health benefits most notably in the treatment of common diseases such as irritable bowel syndrome (Enck et al., 2008) and diarrhoea (Wunderlich et al., 1989), while health-promoting properties such as hypocholesterolaemic (Guo et al., 2015) and immunostimulatory (Molina et al., 2015) effects have also been observed.

Moreover, enterococci have also demonstrated the ability to generate bioactive peptides during milk fermentations owing to the possession of highly proteolytic extracellular enzymes. *E. faecalis* secretes two dominant proteases, gelatinase and serine protease (Thomas et al., 2008). Current literature suggests that gelatinase (*gelE*) is responsible for casein degradation, while strains containing serine

*Abbreviations:* ACE, Angiotensin Converting Enzyme; ANOVA, one-way Analysis of Variance; ATCC, American Type Culture Collection; BHI, Brain Heart Infusion; BLAST, Basic Local Alignment Search Tool; CITCC, Cork Institute of Technology Culture Collection; DPC, Dairy Products Culture Collection; KAA, Kanamycin Aesculin Azide Agar; KSMEA, Kanamycin Skim Milk Aesculin Azide Agar; LAB, lactic acid bacteria; OAA, Oxolinic Acid Aesculin Azide Agar; RSM, Reconstituted Skim Milk SMA; Skim Milk Agar SPSS, Statistical Package for Social Sciences.

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protease (*sprE*) do not appear to hydrolyse casein (Gútiez et al., 2014; Maharshak et al., 2015). Gútiez et al. also showed that those strains expressing gelatinase generated peptides when grown in bovine skim milk (BSM) which had angiotensin-converting enzyme (ACE) inhibitory activity (Gútiez et al., 2014). Therefore, these peptides have the potential to be used as food additives as they contribute to the maintenance of normal blood pressure by preventing hypertension, thereby improving the health of the consumer (Gútiez et al., 2013). Contrastingly, it is now understood that proteases can also play a role in the virulence of enterococci, particularly gelatinase which has been shown to increase pathogenicity in an animal model (Franz et al., 2003; Kayser, 2003; Singh et al., 1998). Enterococci are now considered among the most proteolytic bacteria to date and show higher proteolytic activities than other LAB (Bhardwaj et al., 2008; Centeno et al., 1996; Tsakalidou et al., 1994). Consequently, the isolation and identification of proteolytic enterococci is becoming of increasing importance and interest.

Current microbiological practices require a two-step procedure when screening for presumptive proteolytic enterococci. First, enterococci must be isolated from a chosen environmental niche. Several selective and/or differential media have been developed for this purpose and are extensively reviewed in the literature (Domig et al., 2003; Weiss et al., 2005). Among others, Kanamycin Aesculin Azide Agar (KAA) is commonly utilised, particularly for the isolation and enumeration of enterococci from dairy and other foods. The selective pressures of the medium, coupled with aesculin hydrolysis, enable the effective enumeration of enterococci while supressing the growth of the majority of other microbes (Domig et al., 2003). Following isolation of enterococci, assays are applied to assess the proteolytic ability of isolates. The conventional method for initially determining the proteolytic activity of a microorganism has employed cultivation on skim milk agar (SMA). Proteolytic bacteria hydrolyse casein to form soluble nitrogenous compounds which is indicated by a clear zone in the agar surrounding the colonies (Frazier and Rupp, 1928). Therefore, current screening practices can be time consuming, requiring initial isolation of enterococci using KAA followed by an assessment of proteolytic activity using SMA.

Recognizing the limits of currently used screening methods and the increasing interest in enterococci which display proteolytic capabilities, it is desirable to develop a quicker, more convenient and cost effective method to isolate proteolytic enterococci. This study aimed to develop a method, utilising Kanamycin Skim Milk Aesculin Azide Agar (KSMEA), for the isolation of presumptive proteolytic enterococci in one-step and to ensure that the selectivity of commercialised selective/differential media for enterococci was retained while proteolysis was clearly identifiable.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The bacterial strains used in this study (Table 1) were obtained from the American Type Culture Collection (ATCC), the Dairy Products Culture Collection (DPC, Teagasc Food and Research Centre, Moorepark, Fermoy, Co. Cork) and the Cork Institute of Technology (CIT) Culture Collection (CITCC, Cork Institute of Technology, Bishopstown, Co. Cork). Bacterial strains were recovered from −80 °C stocks and routinely cultured in Brain Heart Infusion (BHI) medium (LabM, Heywood, Lancashire, United Kingdom) aerobically at 37 °C. *Lactococcus lactis* and *Lactobacillus brevis* were propagated aerobically at 30 °C.

### 2.2. Media preparation

BHI Agar (LabM; a general purpose complex medium to support the growth of all strains) and Kanamycin Aesculin Azide Agar (KAA, Merck KGaA, Darmstadt, Germany; selective medium for enterococcus; concentration of Kanamycin - 20 mg/l) were prepared according to manufacturer's instructions.

**Table 1**
Bacterial strains used in this study.

| Species | Source | Aesculin hydrolysis | Protein hydrolysis |
|---|---|---|---|
| *Escherichia coli* | ATCC 25922 | Negative | N/A |
| *Pseudomonas aeruginosa* | CITCC K01 | Negative | N/A |
| *Salmonella enterica* | CITCC K02 | Negative | N/A |
| *Bacillus subtilis* | ATCC 6633 | Positive | N/A |
| *Staphylococcus aureus* | CITCC K03 | Negative | N/A |
| *Lactococcus lactis* | CITCC K04 | Negative | N/A |
| *Streptococcus bovis* | CITCC K05 | Positive | N/A |
| *Lactobacillus brevis* | CITCC K06 | Negative | N/A |
| *Enterococcus faecalis* | ATCC 29212 | Positive | Negative |
| *Enterococcus faecalis* | DPC 5203 | Positive | Negative |
| *Enterococcus faecium* | DPC 4983 | Positive | Negative |
| *Enterococcus faecalis* | DPC 3891 | Positive | Negative |
| *Enterococcus faecalis* | DPC 5186 | Positive | Negative |
| *Enterococcus faecalis* | DPC 1144 | Positive | Negative |
| *Enterococcus casseliflavus* | DPC 4993 | Positive | Negative |
| *Enterococcus casseliflavus* | DPC 4960 | Positive | Negative |
| *Enterococcus faecalis* | DPC 5209 | Positive | Positive |

ATCC: American Type Culture Collection; CITCC: Cork Institute of Technology Culture Collection; DPC: Dairy Products Culture Collection, Teagasc.
N/A: Not applicable, strains used for assessment of selectivity only.
*E. faecalis* – Casein hydrolysis is due to the presence of the *gelE* gene encoding gelatinase (Gútiez et al., 2014; Maharshak et al., 2015).

To prepare Skim Milk Agar (SMA), 10% (w/v) Reconstituted Skim Milk (RSM) Powder (Glanbia, Fermoy, Cork, Ireland) was sterilized at 110 °C for 10 min and aseptically added to 5% (w/v) sterile agar (LabM) held at 50 °C in a 1:1 ratio. The solution was gently mixed to homogeneity ensuring that the formation of air bubbles was avoided and then poured into sterile petri dishes.

Kanamycin Skim Milk Aesculin Azide Agar (KSMEA) was prepared by combining KAA with RSM. Double strength KAA was sterilized at 121 °C for 15 min and mixed with sterile 10% (w/v) RSM in a 1:1 ratio.

### 2.3. Evaluation of the selective properties of KSMEA

To evaluate the selective properties of KSMEA, eight non-proteolytic *Enterococcus* strains and eight non-*Enterococcus* strains were selected in accordance with control strains used for commercialised KAA (Table 1). The latter consisted of *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Lactococcus lactis*, *Streptococcus bovis* and *Lactobacillus brevis*. Proteolysis was not of concern when evaluating the selective performance of KSMEA relative to KAA therefore the enterococcal strains used were non-proteolytic (P. Simpson et al., un-published data). 1 μl of overnight cultures in BHI broth were spot inoculated onto BHI agar, KAA agar and KSMEA agar and incubated at 37 °C for 24 h. Following incubation, the media were analysed for growth and aesculin hydrolysis by the bacterial strains.

### 2.4. Assessment of proteolysis and recovery of enterococci on KSMEA

To evaluate the capability of KSMEA to isolate and distinguish between proteolytic and non-proteolytic enterococci, *E. faecalis* DPC 5209 (DPC, Teagasc – confirmed as proteolytic by observation of growth and zone of hydrolysis on SMA and also by the o-phthalaldehyde assay - P. Simpson et al., unpublished data) and *E. faecalis* ATCC 29212 (confirmed as non-proteolytic) were used. Both stains were grown in BHI broth at 37 °C for 18 h. The cultures were serially diluted 10-fold in sterile ringers (LabM) and spread plated onto BHI agar, SMA, KAA and KSMEA. This was performed in triplicate and average results reported. Growth, proteolysis and aesculin hydrolysis was assessed following incubation at 37 °C for 48 h. Colony counts were recorded for both cultures on all media after the incubation period to assess the recovery of enterococci on KSMEA.