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Comparison of Selective Media for the Enumeration of Probiotic Enterococci from Animal Feed**

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Summary

The project »Methods for the Official Control of Probiotics Used as Feed Additives« has been undertaken to develop and validate methods for the selective enumeration and strain identification of six probiotic microorganism genera (enterococci, lactobacilli, bifidobacteria, pediococci, bacilli and yeast). A diversity of media has been used for the detection, isolation and enumeration of enterococci. Aiming at the selective enumeration of enterococci (mainly *Enterococcus faecium*) present in probiotic animal feeds, either as a single component or in combination with other microorganisms, an extensive screening of published methods for culturing and enumerating enterococci was carried out. A collection of enterococcal strains used as probiotics in animal feeds and of isolates as well as reference strains from culture collections was established. Moreover, selected strains of lactobacilli, pediococci and streptococci were included for reference purposes. Based on a multitude of publications, twelve commercially available media were selected for testing and then compared with regard to their usefulness and selectivity. Bile esculin azide (BEA) agar showed good selectivity and pronounced growth of most enterococcal strains. Good reproducibility and electivity (esculin hydrolysis) as well as no influence of the feed matrix on the colony counts and a simple preparation procedure formed the basis for the proposed enumeration protocol. This work formed the basis for the enumeration protocol that was adapted to ISO format and validated in a collaborative study involving twenty laboratories from twelve European countries.

Key words: enterococci, media, enumeration, bile esculin azide agar, probiotic feed

Introduction

Microorganisms such as enterococci have been used as probiotics in feeds for several years. Their positive effect on the gut flora results in an improved health status, especially for young animals, but also in an improved animal performance such as growth or feed conversion rate (*1*). The application of microorganisms in feeds is regulated under the scope of Council Directive 70/524/EEC (*2*), which is based on the main principles of pre–market authorisation, positive list principle, and thorough risk assessment (*1*). The products (which are either microorganisms and their preparations, or compound feeds into which microorganisms have been incorporated) must include in their labelling the identification of the strain(s) according to the recognised international code of nomenclature, the deposit number of the strain(s) according to the recognised international code of nomenclature, the deposit number of the strain(s) and the number of colony forming units per

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gram, provided that it is measurable by an official or scientifically valid method (*3*). Due to the importance of enterococci in different foods, feeds, clinical and environmental samples, numerous media have been used for the detection, isolation and enumeration (*4*). This study was performed within an EU project initiated by a Dedicated Call and was based on the regulations of the Council Directive 70/524/EEC (*2*) concerning additives in feeds and the application of probiotics in feeds. According to the Official Journal of the European Communities C 263/3 (*5*), eleven genera of bacteria, three genera of yeast and one mould have been approved for temporary use within specified member states and are in line with the provisions of Council Directive 93/113/EC (*3*).

Twelve media were selected based on a literature search (*4*) and tested with a set of strains. The species were selected based on their involvement in probiotic animal feeds as outlined in the Official Journal of the European Communities C 263/3 (*5*).

Material and Methods

Test strains

The strain set used for the selectivity test consisted of twenty-eight enterococci, eleven lactobacilli, six pediococci and one *Streptococcus thermophilus* strain (see Table 1). Lactobacilli, pediococci and *S. thermophilus* were chosen as they are authorised by the EU to be incorporated in feeds, and are so closely related to enterococci that they might interfere with their enumeration.

Culturing of test strains

The test strains used were subcultured as follows: enterococci were grown in 10 mL of brain heart infusion broth (BHI, CM 225, Oxoid) under aerobic conditions for 24 h at 37 °C. Lactobacilli were grown in MRS broth (1.10661, Merck) under anaerobic conditions (anaerobic chamber; atmosphere: N_2 80 %, CO_2 10 % and H_2 10 %) for 24 h at 37 °C. Trypticase soy broth (TS, CM 129, Oxoid) supplemented with 3 g/L of yeast extract (1.037753, Merck) was used for subculturing of the *S. thermophilus* strain under anaerobic conditions at 32 °C for 24 h and for pediococci under the same conditions for 48 h.

Selective media

The tested selective media were prepared according to the author's or manufacturers' guidelines. After sterilisation the media were cooled to 50 °C and plates were prepared by pouring 17 mL of sterile medium into a sterile Petri dish. All media used are described below, including important details of their preparation.

Bile esculin (BE) agar (CM 888, Oxoid; 44.5 g/L), which was autoclaved for 15 min at 121 °C, was applied. Additionally, two bile esculin azide (BEA) agars from different manufacturers were tested: enterococcosel (ECSA) agar (4312205, Becton-Dickinson; 56 g/L, which was autoclaved for 15 min at 121 °C) and D-coccosel (D-Cocc) agar (51025, bioMérieux; 56 g/L, which was autoclaved for 15 min at 121 °C).

Table 1. List of test strains

Strain Code	Genus – species – subspecies	Other code
En1	*Enterococcus faecalis*	DSM 2570
En2	*E. faecalis*	DSM 20478
En3	*E. faecium*	DSM 2918
En4	*E. faecium*	DSM 20477
En5	*E. durans*	DSM 20633
En6	*E. gallinarum*	DSM 20628
En7	*E. faecium*	Own isolate
En8	*E. faecium*	Own isolate
En9	*E. faecium*	Own isolate
En10	*E. faecium*	Own isolate
En11	*E. faecium*	DSM 3320
En12	*E. faecalis*	DSM 2981
En12A	*E. faecalis*	ATCC 14506
En13	*E. durans*	ATCC 6056
En14	*E. faecium*	ATCC 27270
En15	*E. faecalis*	ATCC 27274
En16	*E. faecium*	DSM 2146
En17	*E. faecium*	DSM 6177
En18	*E. avium*	DSM 20679
En19	*E. casseliflavus*	DSM 20680
En20	*E. hirae*	DSM 20160
En21	*E. malodoratus*	DSM 20681
En22	*E. mundtii*	DSM 4838
En23	*E. raffinosus*	DSM 5633
En24	*E. faecium*	DSM 7134
En25	*E. faecium*	NCIMB 10415
En26	*E. faecium*	NCIMB 11181
En27	*E. faecium*	NCIMB 30096
En28	*E. faecium*	Own isolate
En30	*E. faecium*	ATCC 19434
Lb1	*Lactobacillus acidophilus*	DSM 20079
Lb7	*L. amylovorus*	DSM 20531
Lb12	*L. rhamnosus*	DSM 20021
Lb13	*L. reuteri*	DSM 20016
Lb14	*L. plantarum*	Own isolate
Lb17	*L. fermentum*	ATCC 9338
Lb20	*L. casei*	Own isolate
Lb21	*L. reuteri*	Own isolate
Lb22	*L. casei*	DSM 20011
Lb23	*L. plantarum*	DSM 20174
Lb28	*L. rhamnosus*	DSM 7133
Sc1	*Streptococcus thermophilus*	DSM 20617
Pd1	*Pediococcus acidilactici*	DSM 20248
Pd2	*P. acidilactici*	DSM 20238
Pd3	*P. damnosus*	DSM 20331
Pd4	*P. parvulus*	DSM 20332
Pd5	*P. dextrinicus*	DSM 20335
Pd6	*P. pentosaceus*	DSM 20336
Pd7	*P. pentosaceus*	ATCC 43200
Pd8	*P. inopinatus*	DSM 20285

Cephalexin aztreonam arabinose (CAA) agar (according to Ford *et al.* (*6*)) consists of Columbia agar base 40 g/L, arabinose 10 g/L and phenol red solution 3.6 mL/L, *m/V*=2. The pH value was adjusted to pH=7.8 and the medium was autoclaved for 20 min at 114 °C (0.7 bar). After the medium had reached 50 °C, fresh sterile solutions of aztreonam and cephalexin were added to reach the final concentrations of 75 and 50 mg/L, respectively.

Chromocult enterococci (CE) agar (1.10294, Merck; Chromocult enterococci broth 18 g/L and agar 14 g/L) was autoclaved for 15 min at 121 °C.

Citrate azide Tween carbonate (CATC) agar (1.10279, Merck; 56 g/L) was autoclaved for 15 min at 121 °C. When the medium had reached approximately 50 °C, filter-sterilised solutions were added: 20 mL of sodium-hydrogen carbonate *m/V*=10, 10 mL of 2,3,5-triphenyl tetrazolium chloride (TTC) solution *m/V*=1 and 4 mL of sodium azide solution *m/V*=10.

mE (mE) agar (0333-17, Difco; 72.1 g/L) was autoclaved at 121 °C for 15 min. When the medium had reached a temperature of approximately 50 °C, filter-sterilised solutions were added: 15 mL of TTC *m/V*=1 and 0.24 g of nalidixic acid.

Fluorescent gentamicin thallous carbonate (fGTC) agar (according to Littel and Hartman (*7*)) was obtained by suspending CASO agar 40 g, potassium-dihydrogenphosphate 5 g, amylose azure 3 g, galactose 1 g, thallium azure 0.5 g, 4-methyl umbelliferyl-α-D-galactoside 100 mg and 0.75 mL of Tween® 80 in 1 L of distilled water and mixing thoroughly. Then 2.5 mL of gentamicin solution (1 mg/mL) were added and autoclaved for 15 min at 121 °C. When the medium had reached a temperature of approximately 55 °C, 20 mL of filter-sterilised sodium hydrogen carbonate solution (*m/V*=10) were added.

Kanamycin esculin azide (KEA) agar (CM 591, Oxoid; 42.6 g/L) was obtained by adding two vials of kanamycin supplement (SR92, Oxoid), and then autoclaved for 15 min at 121 °C.

KF-streptococcus (KF) agar (CM 701, Oxoid; 76.4 g/L) was autoclaved at 121 °C for 10 min. When the medium had reached 50 °C, 10 mL of filter-sterilised TTC solution (*m/V*=1) were added.

Membrane-filter enterococcus selective (SB) agar (according to Slanetz and Bartley (*8*); 1.05289, Merck; 41.5 g/L) was sterilised by heating in a current of steam in an autoclave without excess pressure for 20 min. When the medium had cooled to approximately 50 °C, 10 mL of filter-sterilised TTC (*m/V*=1) were added.

Merckoplate Barnes (BA) agar (13576, Merck; ready-to-use plates) was used.

Oxolonic acid esculin azide (OAA) agar (according to Audicana *et al.* (*9*)) was obtained by suspending 42.6 g of kanamycin esculin azide agar (CM 591, Oxoid) and 0.25 g of sodium azide in 1 L and then autoclaved for 15 min at 121 °C. Oxolonic acid (0.1 g) was dissolved in 5 mL of 0.1 M NaOH and filter-sterilised. The aliquots of the solution (1 mL of each) were stored at –20 °C. When the medium had reached a temperature of approximately 45 °C, 0.5 mL of oxolonic acid solution were added under aseptic conditions.

Sterile media in Petri dishes, protected against dehydration, were stored for up to two weeks at a temperature of 4 °C.

Evaluation of selective media

Based on a comprehensive literature review (*4*), the cited media were grouped according to their selective components. Already well-evaluated, mainly commercially available media were selected out of the main groups to test their selectivity with a set of enterococci and strains of genera of other lactic acid bacteria. The subcultured strains were loop-streaked onto the agar plates. After incubation under aerobic conditions for 24 h (BE, ECSA, D-Cocc, CAA, CATC, fGTC, KEA, SB, BA, OAA) or for 48 h (CE, mE, KF), the growth of the test strains was evaluated. The colony size was measured (for parameters see Tables 2 and 3), then the growth was described, and digital photographs of the whole plates and close ups (microscope SZH10, Olympus) of single colonies were taken (data not shown).

Comparative testing of three selective media and BHI agar

In order to set up a protocol for the enumeration of enterococci in feeds and feed supplements, the three most selective media (ECSA, mE and CATC) were chosen and included in further trials. Their performance was compared with the non-selective control medium BHI concerning their productivity. The productivity was calculated according to the following formula:

$$P = \frac{N_s^s}{N_0^s} \qquad /1/$$

N_s^s number of colonies of the sought type obtained on selective medium

N_0^s number of colonies of the sought type obtained on control medium

From a liquid culture of the strain *E. faecium* En24 (contained in the feed supplement »Provita – LE«) one loop was transferred into sterile BHI broth, blended and incubated under aerobic conditions for 24 h at 37 °C. A volume of 1 mL of this turbid broth was used to prepare a single series of decimal dilutions up to the dilution of $1:10^7$. The dilutions of $1:10^5$, $1:10^6$ and $1:10^7$ were used to inoculate plates of the tested selective media and BHI (two parallel plates per dilution) using the surface plating method. The inverted plates were incubated under aerobic conditions for 24 h at 37 °C. The colony-forming units (CFU) were determined right after the incubation.

The arithmetic means of the colony counts of the plates with the optimum dilution (between 5 and 300 colony-forming units per plate) and the productivities were calculated. The experiment was repeated with modifications (mE agar was no more included, the dilutions of $1:10^6$, $1:10^7$ and $1:10^8$ were applied for inoculation, three plates were inoculated at the same time for the dilution of $1:10^7$ and two plates for each of the other dilutions).

Table 2. Growth intensities of enterococci on tested media

	Test strains	Tested media												
		KEA	OAA	ECSA	D-Cocc	BE	KF	CATC	SB	BA	mE	CAA	fGTC	CE
En1	*E. faecalis*	++	++	++	++	++	++	++	+	++	++	+	++	+
En2	*E. faecalis*	++	++	++	++	++	++/+++	++	+	++	+	++	+++	+
En3	*E. faecium*	++/+++	++	++	++/+	++	+	+	(+)	(+)	++	(+)	++	++
En4	*E. faecium*	++	++	++	++	++	++	++/+	++	++/+	++	++	+++	+
En5	*E. durans*	+	++	+	+	++	+	(+)	(+)	(+)	+	++/+	++/+	+
En6	*E. gallinarum*	++/+	++/+	+	+	++	+	(+)	(+)	+	+	(+)	+++	+
En7	*E. faecium*	++/+++	++	++	++	++/+	++/+	+	+	+	++	++	+++	++
En8	*E. faecium*	++/+++	++	++	++	++	++	+	+	+	++	(+)	++	++
En9	*E. faecium*	++	++	++	++	++/+++	+++	++	+	(+)	++	(+)	++	++
En10	*E. faecium*	++/+++	++	++	++	++	+++	++	+	(+)	++	+	++	++
En11	*E. faecium*	++/+++	++	++	++	++	+++	+	++	++/+	+	++	++	++/+
En12	*E. faecalis*	++/+++	++	++	++	++/+++	++/+++	++	+	++	++/+	++/+	++/+++	++
En12A	*E. faecalis*	++	++	++/+++	++	++/+++	++/+++	+/(+)	+	++	++	++	+++	++
En13	*E. durans*	+	+	++/+	+	++/+	+	(+)	+/(+)	+	+	+	++	+
En14	*E. faecium*	++	++/+	++/+	++	+	+++	+	+	++/+	++/+	+	++	+
En15	*E. faecalis*	+++	++/+	++/+++	++	+	++	++	+	++	++	++	++	++
En16	*E. faecium*	++	++	++/+	++	++	+++	++/+	+	+	++/+++	(+)	++	++/+
En17	*E. faecium*	++	++	++	++	++	++/+	++/+	+	+	++	++	++	++/+
En18	*E. avium*	+	+	+	+	++	+	+	+/(+)	+	+	(+)	++	++/+
En19	*E. casseliflavus*	++/+	++/+	+	+	++	++	+	+/(+)	+	+	(+)	++	+
En20	*E. hirae*	++/+++	++	++	++/+	++	++	+	+	++/+	++	++	+++	++/+
En21	*E. malodoratus*	(+)	+/(+)	+	+	+	+	(+)	(+)	+	(+)	(+)	++/+	+
En22	*E. mundtii*	++	++	++	++	++/+	++	(+)	+	++	+	(+)	++	++/+
En23	*E. raffinosus*	++/+	(+)	(+)	(+)	++/+	(+)	(+)	(+)	(+)	(+)	+	++/+	+/(+)
En24	*E. faecium*	++	+++	++/+++	++	++	+++	+	++/+	++/+	++	++/+	+++	++/+++
En25	*E. faecium*	++	++	++	++	++	++	++	++	+	++	++/+	++	++
En26	*E. faecium*	+++	++/+++	+	++	++	+++	++	++	++/+	++	++/+	++/+++	++
En27	*E. faecium*	++	++	++	++	++	++	(+)	++	+	++	++	++	++
En28	*E. faecium*	++/+++	++	+	++	++	++	+	+	++/+	++	(+)	++	++/+

Parameters used to describe the growth intensity of the tested strains: **+++,** large colonies, >1 mm in diameter; **++,** medium size colonies, 1 mm in diameter; **+**, small colonies, <1 mm in diameter; (+), pinpoint sized colonies

Comparative testing of BEA agar (ECSA) with BHI agar

The selective medium BEA agar in the formulation of ECSA agar was further tested concerning its productivity, selectivity and electivity in comparison with BHI medium. Furthermore, possible influences on the results of the used methods (surface-plating and pouring), and possible matrix influences (*e.g.* feed powder or feed flakes in comparison with pure cultures) were tested.

A mass of 10 g of the feed supplement »Provita – LE« (containing *E. faecium* En24 and *L. rhamnosus* Lb28, see Table 1) were weighed into a sterile plastic bag and 90 mL of sterile NaCl solution m/V=0.85 (at 4 °C) were added. The mixture was homogenised in a Stomacher-blender for 1 min and then stored for 15 min at 4 °C to allow coarse material to sediment. A volume of 1 mL of the supernatant was transferred into a tube containing 9 mL of sterile NaCl solution at a temperature of 4 °C, followed by mixing the suspension for 5 s (Vortex). The same procedure was applied to all further decimal dilutions. At the same time, a second series was prepared accordingly. From each series the three dilution steps of $1{:}10^7$, $1{:}10^8$ and $1{:}10^9$ were selected for the inoculation of the plates, because they had shown acceptable results of colony counts in the preceding trials. An aliquot (0.1 mL) of each dilution was inoculated to each of the two media using both methods (surface plating and pouring), resulting in four different groups per dilution (ECSA-surface, ECSA-pouring, BHI-surface, BHI-pouring). Per group, 10 plates were inoculated at the same time. The inverted plates were incubated under aerobic conditions for 24 h at 37 °C. Right after the incubation the number of CFU/g of presumptive enterococci of each plate displaying 5 to 300 colonies was calculated.

The colony counts comprised the following types of colonies: BHI-pouring (lentil-shaped white colonies, $2r \sim 1$ mm), BHI-surface (circular, entire, white colonies, $2r \sim 1$

Table 3. Growth intensities of the other genera of lactic acid bacteria on the tested media

	Test strains	Tested media												
		KEA	OAA	ECSA	D-Cocc	BE	KF	CATC	SB	BA	mE	CAA	fGTC	CE
Lb1	*L. acidophilus*	–	–	–	–	–	–	–	–	(+)	–	–	–	–
Lb7	*L. amylovorus*	(+)	–	–	–	(+)	–	–	–	(–)	–	–	(–)	–
Lb12	*L. rhamnosus*	(+)	–	–	–	(+)	–	–	–	(+)	–	(+)	(+)	–
Lb13	*L. reuteri*	(+)	+	(+)	(+)	(+)	(+)	–	(+)	(+)	–	(+)	–	(+)
Lb14	*L. plantarum*	(+)	–	–	–	–	–	–	–	(+)	–	–	(+)	–
Lb17	*L. fermentum*	–	–	–	–	–	–	–	–	(+)	–	(+)	(+)	–
Lb20	*L. casei*	(+)	–	(+)	–	(+)	–	–	–	(–)	–	(+)	(+)	–
Lb21	*L. reuteri*	–	–	–	–	–	–	–	–	(–)	–	(+)	–	–
Lb22	*L. casei*	(+)	(+)	–	–	(+)	(+)	–	–	(–)	–	(+)	(+)	(+)
Lb23	*L. plantarum*	(+)	+	(+)	(+)	(+)	+	–	(+)	(+)	(+)	(+)	(+)	(+)
Lb28	*L. rhamnosus*	(+)	–	–	–	(+)	–	–	–	(+)	–	(+)	(+)	–
Sc1	*S. thermophilus*	–	–	–	–	–	–	–	–	–	–	–	(+)	–
Pd1	*P. acidilactici*	(+)	+	(+)	(+)	(+)	+	–	(+)	(+)	(+)	(+)	(+)	(+)
Pd2	*P. acidilactici*	(+)	+	(+)	(+)	(+)/+	+	–	(+)	(+)	(+)	(+)	(+)	(+)
Pd3	*P. damnosus*	–	–	–	–	–	–	–	–	(+)	–	(+)	–	–
Pd4	*P. parvulus*	(+)	–	(+)	–	(+)	–	–	–	(+)	–	(+)	(+)	–
Pd5	*P. dextrinicus*	(+)	+	(+)	(+)	(+)	+	–	(+)	(+)	(+)	(+)	(+)	(+)
Pd6	*P. pentosaceus*	(+)	+	(+)	–	(+)	(+)	–	(+)	(+)	(+)	(+)	(+)	(+)/+
Pd7	*P. pentosaceus*	(+)	+	(+)	(+)	(+)	(+)	–	(+)	(+)	(+)	(+)	(+)	(+)
Pd8	*P. inopinatus*	–	–	–	–	–	–	–	–	(+)	–	(+)	–	–

Parameters used to describe the growth intensity of the tested strains: **+++,** large colonies, >1 mm in diameter; **++,** medium size colonies, 1 mm in diameter; **+,** small colonies, <1 mm in diameter; (+), pinpoint sized colonies; (–), questionable growth; –, no growth

mm), ECSA-pouring (lentil-shaped white colonies, from a distance appearing as black due to esculin hydrolysis, and surrounded by dark brown to black halos in the medium, 2*r*~1 mm), and ECSA-surface (circular, entire, white colonies, surrounded by dark brown to black halos of hydrolysis in the medium, 2*r*~1 mm).

For the examination of the method with pure strains, one loop was transferred from a liquid culture of the corresponding strain (*E. faecium* En24) into 10 mL of sterile BHI broth and incubated under aerobic conditions for 24 h at 37 °C. A volume of 1 mL of this culture was used to set up a decimal dilution series (NaCl *m/V*=0.85 at 4 °C). For a second dilution series, this step was repeated. The dilutions of $1:10^5$, $1:10^6$ and $1:10^7$ were selected for inoculation of the plates. Further procedure of this experiment was carried out according to the protocol used for the examination of feeds described above.

The same procedure as described above was also applied to the feed sample »F1« and its specific *E. faecium* strain En30 (see Table 1), but using all diluents at room temperature and not at 4 °C. The chosen dilutions were $1:10^5$, $1:10^6$ and $1:10^7$ for both, the feed sample and the pure culture.

The arithmetic mean, the standard deviation and the relative coefficient of variation, CV, were calculated for each set of ten parallel plates according to Sachs (*10*).

These coefficients of the different sets of samples were compared. For further analysis, an LSD test was conducted according to Sachs (*10*). For each strain/feed additive or strain/feed sample the LSD test was applied for both of the two methods (pouring and surface-plating) of one series. In order to do so, the arithmetic means of groups of the same sample size were arranged according to their value in declining succession. The differences between each two adjoining values were calculated.

The differences between the adjoining values were compared with the calculated LSD value. When the LSD value showed a larger number than the value calculated for the difference, the null-hypothesis (the identity of the two averages) could not be dismissed.

The productivity P was calculated for each set of samples and each method as well as for the feed sample/strain as described above.

Provisional standard for the enumeration of enterococci in feeds

Because of the results of these evaluations, a draft for a provisional standard for the enumeration of enterococci in feeds and feed additives was proposed and validated (*11*).

Results

Evaluation of selective media

The growth intensities and colony sizes obtained on the tested media were encoded and are given in Tables 2 and 3. In detail, the following results were obtained:

Bile esculin (BE) agar

On bile esculin (BE) agar, enterococci grew as colonies of small to large size and white colour, only *E. casseliflavus* exhibited light brown and *E. mundtii* yellow pigmented colonies. The colonies of all strains were glistening. With the exception of *E. avium*, strong hydrolysis of esculin was observed. Seven strains of lactobacilli and six strains of pediococci grew on BE medium. *L. plantarum* could not be distinguished macroscopically from enterococci, due to its ability to hydrolyse esculin and its small colony size.

Bile esculin azide (ECSA) agar

This medium was one of the tested formulations of bile esculin azide (BEA) agar. Most of the *E. faecium* strains grew on ECSA forming medium to large size colonies. The colonies of *E. casseliflavus* were brown and the colonies of *E. mundtii* were yellow. All enterococci with the exception of *E. gallinarum* exhibited strong hydrolysis of esculin. Three *Lactobacillus* and six *Pediococcus* strains displayed minimal growth under the given conditions. The strains *L. plantarum*, *P. dextrinicus* and *P. pentosaceus*, which showed strong hydrolysis of esculin, could be distinguished from enterococci by their colony size.

Bile esculin azide (D-Cocc) agar

This medium was the second of the tested formulations of bile esculin azide (BEA) agar. Few enterococci showed poor growth (small colonies), but most grew on D-coccosel agar as medium-sized colonies, exhibiting strong hydrolysis of esculin. *E. mundtii* showed light brown colonies. Two strains of lactobacilli and four strains of pediococci were able to grow under the specified conditions, but did not hydrolyse esculin. Only *L. plantarum* exhibited strong esculin hydrolysis, and could be distinguished from enterococci by its colony size.

Cephalexin aztreonam arabinose (CAA) agar

On CAA agar *E. faecium* grew as white colonies of pinpoint to medium size. *E. mundtii* displayed yellow pigmented colonies. For some enterococci acidification was observed. Growth was detected for eight strains of lactobacilli and seven strains of pediococci. They all grew as pinpoint colonies, some exhibiting acidification. Enterococci, therefore, could not be distinguished macroscopically from lactobacilli and pediococci on CAA medium.

Chromocult enterococci (CE) agar

On this agar all enterococci grew as blue colonies of pinpoint to large size. Three strains of lactobacilli and five strains of pediococci exhibited growth on this medium. *L. casei*, *L. plantarum*, *P. dextrinicus* and two strains of *P. pentosaceus* could not be distinguished from enterococci macroscopically.

Citrate Tween carbonate azide (CATC) agar

On CATC medium, enterococci appeared as colonies of various sizes (pinpoint to medium) and colours (rosy to dark red). Only enterococci were able to grow on this medium, but on account of the small colony sizes the plates were difficult to evaluate. Under the given test conditions, CATC medium displayed the best selectivity.

mE (mE) agar

Enterococci grew as pinpoint to large size colonies of rosy to dark red colour on mE medium, some colonies were thus too small for colony counting. The colonies of the type strains of *E. durans* and *E. gallinarum* were surrounded by light red halos. The type strain of *L. plantarum* and five strains of pediococci grew on mE medium. They were distinguishable from enterococci.

Fluorescent gentamicin thallous carbonate (fGTC) agar

Most enterococci grew on fGTC medium as white colonies. *E. casseliflavus* and *E. mundtii* exhibited yellow pigmented colonies. For some enterococci fluorescence was observed. Seven strains of lactobacilli, six pediococcal strains and *S. thermophilus* grew on fGTC medium. All these strains formed pinpoint-sized colonies and could therefore be distinguished from enterococci.

Kanamycin esculin azide (KEA) agar

All enterococci grew on KEA medium as white colonies of pinpoint to large size except *E. mundtii*, which exhibited yellow colony colour. For two strains of *E. faecalis* and for *E. mundtii* dark centres of the colonies were observed. With the exception of *E. avium* and *E. malodoratus*, all enterococci exhibited strong hydrolysis of esculin. Eight strains of lactobacilli and six strains of pediococci grew on KEA medium. However, they could not be distinguished from enterococci macroscopically.

KF (KF) agar

Enterococci grew as pinpoint to large size colonies on KF medium. The colour of the colonies ranged from pink to dark red. For the type strains of *E. faecalis* and *E. hirae* red and pink halos of the colonies, respectively, could be observed. Some strains of *E. faecium* formed colonies with light zones. Acidification of the medium surrounding the colonies was observed by the change in colour of the indicator dye to yellow. Three strains of lactobacilli and five strains of pediococci grew on KF medium, of which only *P. dextrinicus* could not be distinguished macroscopically from enterococci.

Slanetz Bartley (SB) agar

Enterococci grew on SB medium as rosy to dark red colonies of pinpoint to medium size. Colonies of several strains of *E. faecium* were surrounded by light peripheries. Again, the enterococcal colonies were too small for easy evaluation. Two strains of lactobacilli and five strains of pediococci grew on SB medium. They all formed pinpoint-sized, white colonies. As the *E. faecium* strain En3 showed these characteristics as well, these microorganisms could not be macroscopically distinguished from enterococci.

Merckoplate Barnes (BA) agar

Enterococci grew on BA medium as colonies of pinpoint to medium size. The colony colour ranged from white via rosy to dark red. The colonies of several strains were surrounded by light peripheries. Eleven strains of lactobacilli and seven strains of pediococci grew on BA medium. They all exhibited white colony colour and

pinpoint or small colony size and could therefore not be distinguished from enterococci.

Oxolonic acid esculin azide (OAA) agar

Enterococcal colonies grown on OAA medium were of pinpoint to large size and of white colour, with the exception of *E. mundtii*, which produced yellow colonies. The colonies of the type strain of *E. casseliflavus* and of *E. faecium* En24 showed dark centres. Three strains of lactobacilli and five strains of pediococci grew on OAA--medium. They could not be distinguished macroscopically from enterococci.

Comparative testing of three selective media and BHI agar

On account of the results of this first test series, the three selective media ECSA, CATC and mE were selected for further evaluation. The productivities of all three selective media were close to 100 % (ECSA: 95.2 %, CATC: 111.9 % and mE: 96.4 %). Comparable results were obtained in the second test series (data not shown). Enterococci could be easily recognised on ECSA because they produced a dark brown halo surrounding the colonies due to the hydrolysis of esculin. Plates containing this medium were easy to evaluate: most enterococci formed colonies of medium size (1 mm in diameter) under the specified condition within 24 h. Plates of mE agar were difficult to read because of the very small enterococcal colonies. Under routine laboratory conditions these plates would need to be incubated for at least 48 h, thus this medium was abandoned. CATC plates showed very small colony sizes and lightly coloured colonies as well. Because of the same reasons as for the mE agar, this medium was excluded from further tests.

As this project aimed at developing a standard method for the routine analysis of enterococci in feeds, the main goal was to find a selective medium which is easy to prepare and yields productivity rates comparable to the non-selective BHI medium. Furthermore, much importance was attached to the selectivity and the electivity of the medium, minimising the effort of additional tests for the differentiation and identification of potential enterococci. Of the three selective media evaluated in this test series, ECSA performed best on account of the colony sizes observed and of its good electivity. Thus, this medium was evaluated further in comparison with BHI medium.

Comparative testing of BEA agar (ECSA) with BHI agar

In order to evaluate possible influences on the results of the used methods (surface-plating and pouring), and possible matrix influences on the results (*e.g.* feed powder, feed flakes in comparison with pure cultures) further studies using ECSA and BHI were conducted.

Apart from enterococci no other microorganisms were observed to grow on ECSA under the specified conditions, thus confirming the good selectivity of this medium. The relative coefficients of variation (CV) ranged from 2.62 to 13.41 %, only in three cases exceeding 10 %. This value was influenced by neither the sample nor the medium used nor the method (surface-plating or pouring).

The means of the four different sets of values of each test (ECSA pouring and ECSA surface-plating method, BHI pouring and BHI surface-plating method) were entered into the LSD test. The calculated LSD values ranged between 1.32 and 3.35. For all sets of samples (except En30, series 2) the null-hypothesis had to be dismissed at least once, indicating that the two values belong to two sets of samples. This may indicate that differences exist between the two media and the two methods.

The productivities within a method and a series ranged from 0.1 to 5.6 % for the surface-plating, and from 0.1 to 6.9 % for the pouring method. Higher productivity values were found for the pouring method than for the surface-plating method in three of the four cases, though the differences between the results of the two methods were minute. This may be on account of the uniform exposure of the enterococci to oxygen. For this reason and because of its advantages in routine control, the surface-plating method was chosen for all further examinations.

Provisional standard for the enumeration of enterococci in feeds

Based on these findings a provisional standard protocol (bile esculin azide agar, surface-plating method, aerobic incubation for 24 h at (37±1) °C) was developed. For further method details see Leuschner *et al.* (*11*).

Discussion

Based on an extensive literature review and on the findings of Reuter and Klein (*12*), well-known media were selected and formed the basis of this investigation. For selectivity testing, sets of enterococci and other lactic acid bacteria (LAB) test strains were used. The LAB strains were selected in a way that their genus and species corresponded to the species that were also applied as feed additives. The chosen twelve selective media were evaluated concerning their selectivity for enterococci. The test strains were loop-streaked onto the agar plates. After incubation for 24 or 48 h under aerobic conditions to enhance the selectivity, the growth of the strains was evaluated.

The agar evaluation (Tables 2 and 3) resulted in the selection of three agars for further testing. CATC displayed the best selectivity, but several enterococcal test strains were also inhibited in their growth (small colonies). These findings correspond to those of Ellerbroek (*13*), who compared CATC, BE and SB agars. ECSA and mE agar were chosen because of their good selectivity and the relatively pronounced growth of enterococcal strains. Strains of other genera than enterococci grew only as pinpoint colonies on these two agars and could be easily distinguished from enterococci. The evaluation (BHI as non-selective medium) yielded productivity values close to 100 % for all three media. Under restrictive incubation conditions (24 h, aerobic conditions) enterococcal colonies could be easily distinguished on ECSA agar, whereas the evaluation of the small enterococcal colonies was more difficult on CATC and mE agar.

Based on these findings, BEA (ECSA) agar was chosen for further studies.

This enumeration medium was tested with feed samples and pure cultures of the enterococcal strains contained therein. The results indicated good reproducibility and selectivity for the enumeration based on BEA (ECSA) agar. No influence of the feed matrix or of other LAB strains was detected, because no other tested microorganisms than enterococci were able to grow on ECSA under the specified conditions when evaluating the feed samples. The electivity proved to be very high, because the enterococcal colonies were surrounded by dark brown to black halos of hydrolysed esculin. Based on these findings, the proposed enumeration protocol for enterococci in feeds was developed and the feed samples provided by the coordinator were analysed. The protocol layout was adapted to ISO format.

The proposed enumeration protocol was validated in a collaborative trial. The participating laboratories submitted a high number of results for enumeration of enterococci present as a single component or as part of a mixture with other probiotic LAB microorganisms. The validation study demonstrated good reproducibility and repeatability. Other probiotic microorganisms present in the samples such as yeast, pediococci and lactobacilli were not able to grow on BEA agar. Hence, the enumeration method is recommended as official control method for authorised probiotic enterococci used as feed additives, as reported by Leuschner *et al.* (*11*).

Conclusion

The proposed enumeration method based on bile esculin azide (BEA) agar and the surface-plating method followed by confirmation by api 20 Strep was validated in a collaborative trial. The validation study confirmed the good reproducibility and repeatability. Other probiotic microorganisms (yeasts, pediococci, lactobacilli) present in the samples were not able to grow on BEA. The method was recommended by the project partners (SMT4-CT98-2235: Methods for the official control of probiotics (microorganisms) used as feed additives) for the acceptance for the official control of authorised probiotic enterococci used as feed additives.

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Usporedba selektivnih podloga za identifikaciju probiotičkih enterokoka iz stočne hrane

Sažetak

Projekt »Metode za službenu kontrolu probiotika koji se koriste kao dodaci hrani« pokrenut je da bi se razvili i potvrdili postupci za identifikaciju sojeva 6 rodova probiotičkih mikroorganizama (enterokoki, laktobacili, bifidobakterije, pediokoki, bacili i kvasac).

Za detekciju, izolaciju i popisivanje enterokoka upotrijebljene su razne podloge. Da bi se izolirali enterokoki (uglavnom *Enterococcus faecium*) iz probiotičke stočne hrane (bilo kao pojedinačni sastojak, bilo u kombinaciji s drugim mikroorganizmima) provedena je ekstenzivna provjera objavljenih postupaka za uzgoj i izolaciju enterokoka. Utvrđena je zbirka enterokoka koji se koriste kao probiotici u stočnoj hrani, njihovih izolata, te referentnih sojeva odabranih iz zbirke mikroorganizama. Nadalje, kao referentni uzorci obuhvaćeni su odabrani sojevi laktobacila, pediokoka i streptokoka. Na osnovi mnoštva podataka iz literature odabrano je 12 komercijalno dostupnih podloga koje su zatim uspoređene prema njihovoj upotrebljivosti i selektivnosti. Žuč-eskulin-azid agar (BEA) bio je selektivan i omogućio je značajan rast enterokoknih sojeva. Predloženi postupak izolacije temeljio se na dobroj reproducibilnosti i odabiru BEA-agara (hidroliza eskulina) i jednostavnom postupku priprave, pri čemu sastav stočne hrane nije utjecao na ukupni broj kolonija. Ovaj rad čini osnovu za postupak izolacije i popisivanja enterokoka, prihvaćen u ISO standardu koji je provjeren međulaboratorijskim ispitivanjem u 20 laboratorija iz 12 europskih zemalja.