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Preliminary communication

COMPARISON OF DIFFERENT MEDIA FOR ISOLATION AND ENUMERATION OF YEASTS OCCURRING IN BLUE-VEINED CHEESE

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Comparison of eleven selective media for detecting and enumerating foodborne yeasts in blue-veined cheese showed that rose bengal chloramphenicol agar (RBC), dichloran rose bengal chloramphenicol agar (DRBC), oxytetracycline gentamycin glucose yeast extract agar (OGGY) and dichloran 18% glycerol agar (DG18) were the most efficient. Other examined media failed to be suitable for either inhibiting bacteria and suppressing the spread of moulds or supporting the growth of all yeasts present. Significant differences ($P>0.05$) were obtained on different media, however counts obtained were overlapping on three groups of media. Yeast extract eugenol agar (YEE) medium significantly differed from all others.

Keywords: yeasts, enumeration, media, blue-veined cheese

Lactic acid bacteria and some other bacteria are known to play an essential role in the production of dairy products. Yeasts can be also found frequently within the microbiota of these products. Yeasts may contribute to the ripening and maturation of cheeses (DEVOYOD, 1990; BERGER et al., 1999; MARTIN et al., 2001), but excessive growth of certain yeast species can result in the spoilage of dairy products causing yeasty flavours, gassiness, slime formation and discolouration (FLEET, 1990; VILJOEN & GREYLING, 1995; DEÁK & BEUCHAT, 1996). Moulds are also used as starter cultures in the production of some kind of cheeses, such as *Penicillium camemberti* for Camembert cheese and *P. roquefortii* for blue-veined cheese (LARSEN et al., 1998).

For the purposes of isolation and enumeration of yeasts from foods the use of media which allow the recovery of all kinds of yeast while inhibit bacterial growth and reduce fungal spreading is recommended (KING et al., 1986; FLEET, 1990; DEÁK, 1991; BEUCHAT, 1993). A number of such media exist, however, comparative studies have indicated that none of the currently used media is effective for enumerating yeasts in all foods (DEÁK, 1992; DEÁK et al., 1998; BEUCHAT, 1998). For controlling growth of

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bacteria chloramphenicol, oxytetracycline, gentamycin or some other antibiotics appeared to be equally effective. Chloramphenicol is heat stable and can be added with other ingredients before autoclaving, hence its use is more convenient, while due care is exercised in handling this carcinogenic compound (BECKERS et al., 1986). Various attempts have been made to improve enumeration of yeasts in the presence of filamentous fungi by reducing the colony diameter of spreading moulds. KING and co-workers (1979) described a medium containing chloramphenicol for the inhibition of bacteria as well as dichloran and rose bengal to retard the spreading of moulds. The dichloran rose bengal chloramphenicol agar (DRBC) has become one of the most commonly used isolation media. However, some yeast and mould strains may be inhibited by rose bengal if the medium is exposed to light (CHILVERS et al., 1999).

Several studies have been performed for assessing the role of yeasts in dairy products, and in attempting this several media have been used (FLEET & MIAN, 1987; BARIOLLER & SCHMIDT, 1990; ROHM et al., 1992; JAKOBSEN & NARVHUS, 1996). Recently, WELTHAGEN and VILJOEN (1997) have evaluated ten selective media for their suitability to enumerate yeasts in dairy products. It was found that most antibiotic-supplemented media were superior to acidified media in recovering yeasts from dairy products of neutral pH values. However, all media performed equally well in dairy products of low pH such as cheeses and yoghurt. In the production of mould-ripened cheeses moulds become predominant and can easily overgrow yeasts on enumeration media (ROOSITA & FLEET, 1996). For the purposes of enumerating and isolating yeasts from mould-ripened cheeses, the best suitable medium has yet to be selected or developed. To this end, a comparative study was made to assess and statistically evaluate the performance of various mycological media. Eleven media were tested for their efficiency to support the growth of yeasts in the presence of moulds and bacteria in samples of blue-veined cheese.

1. Materials and methods

1.1. Cheese samples

A roquefort-type cheese (product of Mizo, Hungary) was purchased from a supermarket and analysed immediately. The blue-veined cheese was cut into three sub-samples and ten grams of each portion was homogenized in 90 cm^3 0.1% peptone water with the aid of a Stomacher.

After 3 min settling, further decimal dilutions were prepared up to 10^{-6} g cm^{-3} level in duplicate from each sub-samples.

1.2. Media

Whenever possible, commercially available media were used and prepared according to the manufacturer's instruction. The following media were made: (1) Rose bengal chloramphenicol agar (RBC; Merck), (2) Dichloran Rose bengal chloramphenicol agar

(DRBC; Merck), (3) dichloran 18% glycerol agar (DG18) prepared from a base (Merck) with the addition of chloramphenicol selective supplement (Sigma-Aldrich) (Table 1). Three kinds of malt extract agar were prepared from malt extract broth (Merck), such as (4) malt extract salt agar (MES) supplemented with 4% NaCl and filter sterilized oxytetracycline (100 mg l^{-1}, Fluka), (5) malt extract biphenyl agar (MEP), supplemented with 0.05% (w/v) biphenyl (Fluka), (6) malt extract ox-bile agar (MEOX) supplemented with 0.2% (w/v) ox-bile (Fluka). (7) Oxytetracycline gentamycin glucose yeast extract agar (OGGY) was made from glucose yeast agar base (OGY, Merck) to which filter sterilized oxytetracycline (100 mg l^{-1}, Fluka) and gentamycin sulfate (50 mg l^{-1}, Fluka) were added after autoclaving. (8) Molibdate agar (MOL) was prepared as described by MACLAREN and ARMEN (1958). Briefly, to 100 cm^3 sucrose meat peptone agar base 1.5 cm^3 of 12.5% (w/v) phospho-12-molybdic acid solution (Fluka) was added. (9) Molybdate propionate agar (MOPR) was made from MOL with the addition of 10% (w/v) calcium propionate (Fluka) solution. (10) Yeast extract glucose chloramphenicol agar with oligomycin (YGCO) was made from a commercial YGC base (Merck), whose poured plates were surface supplemented with 0.1 cm^3 filter sterilized oligomycin solution (100 mg l^{-1}, Fluka). (11) Yeast extract eugenol agar (YEE) was also prepared from a commercial yeast extract agar (Merck) supplemented with eugenol (Merck) to give a final concentration of 200 μg cm^{-3}.

All test media were poured into Petri dishes and allowed to dry at room temperature overnight before use. Media prepared in advance were stored at 4 °C until plated.

1.3. Counting

From each medium six plates were prepared (three sub-samples in two duplicates) from each of the three highest dilution (10^{-4}, 10^{-5}, 10^{-6}) by spreading 0.1 cm^3 aliquots of serially diluted samples on the surface of plates. After incubation for 5 days at 25 °C, plates giving 10 to 200 colonies were counted. Colonies were differentiated on the basis of morphology (when in doubt, wet mounts were tested under the microscope) and counts of yeast, mould and bacterial colonies recorded. However, tabulated data are expressed as total CFU g^{-1}.

1.4. Statistical evaluation

After $\log_{10}$ transformation, data were statistically analyzed using a Statgraphics program (Version 5.1; Statistical Graphics Corporation) for two-factorial analysis of variance. Significant differences in mean values of total counts between sub-samples and between media were expressed at $P<0.05$ level.

2. Results and discussion

Total colony counts per g obtained on eleven different mycological media from three sub-samples in duplicates are shown in Table 1.

Table 1. Number of total CFU g^{-1} ($\times 10^5$) counted on various mycological media

Media	Duplicates	Sample 1	Sample 2	Sample 3
RBC	A	118	132	204
	B	95	88	118
DRBC	A	118	150	113
	B	87	94	126
DG18	A	108	136	130
	B	133	114	133
MES	A	114	205	128
	B	122	128	106
MEP	A	68	112	88
	B	74	62	120
MOL	A	60	126	62
	B	64	119	73
OGGY	A	59	132	100
	B	40	69	96
MEOX	A	23	78	97
	B	36	*45	83
MOPR	A	*11	nc	*18
	B	*34	nc	*9
YGCO	A	82	*31	111
	B	75	118	94
YEE	A	*33	nc	*58
	B	nc	*20	*53

All counts are from plates of 10^{-5} dilution except those marked with *. They are counted from plates of 10^{-6} dilution.
nc: not countable

The samples of roquefort-type cheese examined exhibited yeast populations around 10^7 CFU g^{-1}. This population size is similar to those reported from blue-veined cheeses by previous investigators (FLEET, 1990; DEVOYOD, 1990; ROOSITA & FLEET, 1996). Besides yeasts only few mould colonies developed on most selective media, in numbers 2–11 per plate. Hence, with the exception of YEE agar on which a high number of bacterial colonies also developed, the total CFU g^{-1} practically corresponded to yeast counts.

As expected, statistically significant differences were observed in total counts obtained on different media (Table 2). YEE was a clear outlier in that it gave significantly higher counts than all other media. As noted above, this was due to the insufficient antibacterial effect exerted by eugenol included in this medium. The rest of media could be divided into three groups with overlapping statistically significant

differences and no differences within each of the group. One group comprised OGGY, MEOX, MOL and MEP, on these media the mean counts obtained were somewhat less than 10^7 CFU g^{-1}. With the exception of MEP, total counts obtained on these media were significantly less than on those in the other group, MES, MOPR and YEE agar. RBC, DRBC, DG18 and YGCO formed a middle group between the other two, not significantly differing from either groups. The overlapping of plate counts on different media is striking in Fig. 1, showing mean values and pooled 95 percent confidence intervals.

Table 2. Comparison of media using two-factorial analysis of variance

Media	No. of samples	Log_{10} mean count	Homogeneous groups (P>0.05)
OGGY	6	6.88	a
MEOX	6	6.89	a
MOL	6	6.89	a
MEP	6	6.92	ab
DRBC	6	7.05	abc
YGCO	6	7.06	abc
RBC	6	7.08	abc
DG18	6	7.09	abc
MES	6	7.11	bc
MOPR	4	7.19	c
YEE	4	7.57	d

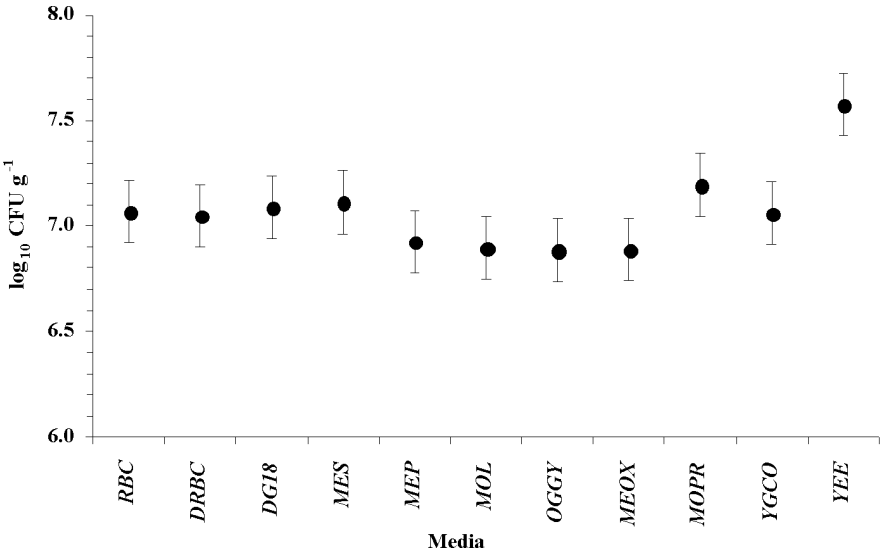

Fig. 1. Mean values of log_{10} plate counts on different media with pooled 95 percent confidence intervals

Disregarding YEE, minor differences were found only between media, although they were statistically significant in some cases. It seems that ox-gall inhibited yeasts to a certain degree in comparison with other malt extract-based media such as MES. The notable difference between MOL and MOPR is difficult to explain, as the latter one is prepared from the former with the addition of calcium propionate. By and large, all media, except MOL and MOPR supported equally the growth of yeasts, however, large differences were observed in the performance of media.

Considering the efficacy in restricting mould growth, the most inhibitory was MEP in that the presence of biphenyl inhibited completely the development of moulds. On the other hand, MOL, MEOX and MOPR did not suppress mould development satisfactorily, hence spreading and overgrowth by moulds rendered the counting of yeast colonies difficult. As to the inhibition of bacteria, calcium propionate, ox-gall and eugenol proved to be inefficient. ELISKASES-LECHNER and PRILLINGER (1996) also found that sodium propionate did not inhibit growth of bacteria, although its inhibitory effect was observed by BOWEN and BEECH (1967). Contrary to previous reports (MOLEYAR & NARASIMHAM, 1992; KIM et al., 1995; VAZQUEZ et al., 2001), eugenol, the main component of clove oil, did not inhibit the growth of bacteria in the final concentration of 200 $\mu g\ ml^{-1}$ at all, so the YEE medium was mostly covered by bacteria. Plates became slimy due to bacterial development and not countable in some cases, or counting was difficult because of the presence of mixed colonies of bacteria and yeasts. ELISKASES-LECHNER & PRILLINGER (1996) using yeast-extract-glucose-chloramphenicol agar supplemented with 100 $\mu g\ ml^{-1}$ oligomycine detected the growth of different kind of moulds, including *Penicillium roquefortii*, but the colony diameter was significantly reduced. In our study YGCO with the same concentration of oligomycine inhibited bacteria, did not restrict enough the growth of moulds. RALE and VAKIL (1984) found that molybdate agar with or without calcium propionate was very useful in isolating yeast from a variety of fruits. According to the present study, molybdate media, MOL and MOPR did not support yeast growth properly and permitted the development only of minute yeast colonies.

In agreement with previous general experience, RBC, DRBC, OGGY, MEP and also DG18 supported well the growth of yeast, while being inhibitory for both bacteria and moulds. Large size of yeast colonies facilitated easy counting, and in addition, DRBC and RBC were also discriminative in colony types (BEUCHAT, 1993; DEÁK & BEUCHAT, 1996).

3. Conclusions

In conclusion, a number of mycological media tested in this study cannot be recommended for use to enumerate yeasts from blue-veined cheese. Among these are MES, MEOX, MOL, MOPR, YEE, YGCO, which failed to inhibit growth of bacteria and/or moulds, or did not support yeast growth appropriately. For convenience of use,

ease of preparation and counting, commercially available media such as DRBC and RBC appears to be a proper choice for the enumeration and isolation of yeasts from blue-veined cheese.

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