DEVELOPMENT OF AN AGAR-MEDIUM (ESCULIN CELLOBIOSE AGAR) FOR THE DETECTION AND ENUMERATION OF LACTOBACILLUS ACIDOPHILUS

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An agar-medium (Esculin cellobiose agar) is presented which enables the detection and quantitative determination of **Lactobacillus acidophilus** in dairy products, especially in fermented milk products. For this purpose the ability of **L.acidophilus** to grow at 40 °C, to ferment cellobiose, and to hydrolyse esculin to esculetine is used. Esculetine forms in combination with Fe (III) ions and olive-green to black complex. For a further differentiation the addition of chlorophenolred was useful. In total 60 strains of lactobacilli, streptococci, and bifidobacteria from 22 species were examined regarding their growth on EC-agar. Only L.acidophilus, L.casei-rhamnosus and L.plantarum could not be differentiated distinctly.

Introduction

There is an increasing trend to use **Lactobacillus acidophilus** instead of or in addition to **L.delbrueckii spp. bulgaricus** as a starter culture for the production of weakly acidified sour milk products [1, 2]. A further field is the application of **L.acidophilus** as a dietary adjunct. This species is suggested to provide several benefits to the digestive system [3, 4, 5]. So **L.acidophilus** is added to various milk products like e.g. "A 38" in Denmark, "Acidophilus Milk" in Sweden and the U.S.A. Finally **L.acidophilus** partly mixed with other lactic acid bacteria and enterococci is offered in pharmaceutical preparations [6, 7]. For the cell count estimation of Lactobacilli normally MRS-agar [8] is recommended. But there is a lack of this medium for a selective enumeration of **L.acidophilus** in products composed of different species of lactic acid bacteria. The purpose of this research was to develop a selective medium for the quantitative determination of **L.acidophilus**.

Material and Methods

Esculin cellobiose agar (EC agar): Tryptone, 20.0 g; yeast extract, 5.0 g; cellobiose, 20.0 g; NaCl, 4.0 g; Na acetate.3 H_2O, 1.5 g; Tween 80, .5 g; esculin, 1.0 g; ammoniumiron (III) citrate (dark brown granulate) .5 g; 6.5 ml of a .2 % chlorophenolred solution (suspend .2 g chlorophenolred in some ml abs. ethanol and fill to 100 ml with distilled water); dissolve the medium by steaming and fill into bottles 80 ml quantities; sterilize at 121 °C for 15 min. After cooling the pH should be 6.6, the colour a weak reddish lilac. For cell count estimation the pour-plate method was used. The agar plates were incubated for 2-3 days at 40 °C in polystyrol bags (plastic bags for petri dishes) evacuated and filled with CO_2 or in anaerobic jars using H_2 + CO_2 generating kits. For control the tested strains were grown parallel to EC agar in lactic agar [9] at optimal growth temperature.

M17 agar: The composition and manufacture of this medium was published by TERZAGHI and SANDINE [11]. Calcium citrate agar: This agar medium was developed by NICKELS and LEESMENT [13] and applied as published in the IDF-Bulletin [14]. Cultures : Following strains have been examined for growth on EC agar: L.acidophilus, DSM[1] 20242, 20077, 20078, 20079, Wiesby[2] 140, 145, 383, 385; L.plantarum, DSM 20199, 20314, Wiesby 264, 384; L.casei-rhamnosus, Wiesby 161, 162, 164, 167; L.casei-casei, Wiesby 160, 287; L.lactis, DSM 20072, Wiesby 148; L.fermentum, Wiesby 238; L.cellobiosus, Wiesby 362; L.bulgaricus, Wiesby 181, 184, 186, 247, 248, 290 ; L.helveticus, Wiesby 177, 178; L.jensenii, Wiesby 360; L.xylosus, DSM 20175; L.curvatus, DSM 20496, 20019; L.brevis, DSM 20054; Pediococcus pentosaceus, Wiesby 274, 275; P.acidilactici, Wiesby 276, 277; Streptococcus faecium, Wiesby 264; S.thermophilus, Wiesby 70, 71, 103, 121, 122, 123; S.cremoris, Wiesby 7, 18, 61, 107, 199; S.lactis, Wiesby 1, 6, 37, 60; Leuconostoc cremoris, Wiesby 342, 343, 437; Bifidobacterium longum, DSM 20219; B.breve, DSM 20213. The test strains were precultured in lactic-broth [9] by optimal temperature.

[1] DSM : German Collection of Microorganisms; Göttingen, FRG
[2] Wiesby : Strain collection; Wiesby, Niebüll, FRG

Results and Discussion

The basic composition of EC agar was supplied by the lactic medium described by Elliker, Anderson and Hannesson [9]. First we tested the necessity of the medium additives for good growth of **Lactobacillus acidophilus.** By this was noticed that the addition of ascorbic acid and gelatine can be neglected. Though Na acetate did not stimulate the growth of **L.acidophilus,** it was used for suppressing the growth of the accompanying bacterial flora [10]. Cellobiose was offered as carbohydrate source, because besides **L.acidophilus** little lactobacilli can ferment this sugar. Further applied was the capacity of **L.acidophilus** to hydrolyse the glucoside esculin to glucose and esculetine. In combination with Fe-ions esculetine forms an olive-green to black complex. The addition of chlorophenolred was useful for further differentiation. The selectivity of EC agar was examined in priority with lactic acid bacteria showing good growth in the temperature range from 37 °C to 45 °C and either to hydrolyse esculin and/or to ferment cellobiose. The results are listed in table 1. **L.jensenii, L.xylosus, L.curvatus, L.brevis, Streptococcus cremoris, S.lactis, Leuconostoc cremoris, Bifidobacterium longum,** and **B.breve** did not propagate on EC agar. Dark brown to brown-black and poorly to strongly erosed colonies surrounded by a dark zone could be found by **L.acidophilus, L.casei-rhamnosus, L.plantarum** and **S.faecium.** Brown to dark brown entire colonies were noticed by **L.cellobiosus** but never surrounded by a dark zone. Detection problems may appear with bottom or surface colonies of **L.acidophilus** showing here spreaded lightly brown coloured colonies. The strain majority of **L.bulgaricus** and **L.helveticus** grew on EC agar with white, wool-like filamentous colonies. **S.thermophilus** showed poor growth on EC agar (transparent pin point

Table 1 : Colony morphology of Lactobacilli, Pediococci, S.faecium and S.thermophilus grown on EC agar (40 °C, 48 h, CO_2-atmosphere)

Species	agar colony morphology	colony colour	colony diameter (mm)	colour of the zone
L.acidophilus	circular, poorly to strongly erosed	dark to black-brown	1,0 - 1,5	dark brown
L.plantarum	circular, poorly to strongly erosed	dark to black-brown	0,5 - 1,5	dark brown to yellowish green
L.casei-rhamnosus	circular, poorly to strongly erosed	dark to black-brown	0,5 - 1,5	grey to brown-black
L.casei-casei	circular, entire	grey to greyish brown	0,5 - 1,5	yellow to greenish yellow
L.lactis	circular, entire	yellow to greenish brown	0,5 - 1,5	yellow to yellowish brown
L.fermentum	circular, entire	greyish brown	0,5 - 1,5	yellowish brown
L.cellobiosus	circular, entire	brown to dark brown	0,5	-
L.bulgaricus	filamentous	white	0,5 - 3,0	-
L.helveticus	filamentous	white	0,5 - 3,0	-
P.pentosaceus	circular, entire	grey/yellowish brown	0,5 - 1,5	brown/greenish yellow
P.acidilactici	circular, entire	yellowish to light brown	0,5 - 1,5	yellowish green
S.faecium	cicular, poorly erosed	dark to black-brown	0,5 - 1,5	brown to yellowish brown
S.thermophilus	punktiform	transparent	< 0,5	

colonies) and should therefore not be calculated. All remaining strains formed entire light brown to grey/greyish-green or yellow/yellowish-brown colonies. These colonies were surrounded by brown-yellow or yellow-green zones. So one can recognize **L.acidophilus, L.casei-rhamnosus, L.plantarum** and **S.faecium** without serious problems. **S.faecium** will be easily differentiated from **L.acidophilus** by microscoping, **L.casei-rhamnosus** and **L.plantarum** by examining the gas formation from gluconate.

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