A Differential Medium for the Enumeration of Homofermentative and Heterofermentative Lactic Acid Bacteria†

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A medium was developed for the differential enumeration of homofermentative and heterofermentative lactic acid bacteria. Essential components of the medium included fructose (14 mM), KH_2PO_4 (18 mM), bromcresol green (as a pH indicator), and other nutrients to support growth. In agar medium, homofermentative colonies were blue to green, while heterofermentative colonies remained white. A total of 21 *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* species were correctly classified with the medium.

Lactic acid bacteria (LAB) are widely distributed throughout nature. They are important in both production and spoilage of acid food products. LAB can be divided into two physiological groups: the heterofermentative LAB, which produce CO_2, lactic acid, acetic acid, ethanol, and mannitol from hexoses, and the homofermentative LAB, which produce primarily lactic acid from hexose (6, 9–11, 14). According to Woolford (15), there is no plating medium which allows rapid differentiation between these two groups of bacteria. Media have been developed which support the growth of these microorganisms, but these media do not distinguish between the two physiological groups (2, 3, 12). Differentiation between homofermentative and heterofermentative LAB has usually been based on the production of CO_2 by heterofermentative LAB (5, 8). Sims (13) designed a selective medium containing fusidic acid which allowed homofermentative lactobacilli of dairy, oral, and vaginal origin to grow but inhibited heterofermentative lactobacilli. We tested this medium with lactobacilli important in vegetable fermentations and found that both homofermentative and heterofermentative lactobacilli were able to grow; thus, differential enumeration was not possible.

Heterofermentative LAB reduce a portion of fructose to mannitol in addition to producing CO_2, lactic acid, and acetic acid when fructose is the sole carbohdyrate source. On the other hand, homofermentative LAB produce 2 mol of lactic acid from any fermentable hexose, including fructose. Since homofermentative LAB produce more acid from a fixed amount of fructose than do heterofermentative LAB, a pH difference can be established. Therefore, we reasoned that the maximum terminal-pH differential between homofermentative and heterofermentative LAB could be produced in a medium by using a limited amount of fructose as the sole carbohydrate source. With this possibility in mind, we developed a medium, which we designated homofermentative-heterofermentative differential (HHD) medium, that allows differential enumeration of homofermentative and heterofermentative LAB.

Streptococcus, *Lactobacillus*, *Leuconostoc*, and *Pediococcus* species were used in this study. All were maintained by weekly transfers through MRS broth (3). For studies with HHD medium, overnight cultures grown in MRS broth were used. Cultures were incubated at 22°C, except when otherwise indicated.

The formulation of HHD medium (Table 1) was based on that of the MD medium of Daeschel et al. (1). By using 0.25% (14 mM) fructose and 0.25% (18 mM) KH_2PO_4 with amino acid sources, a differential of 1 pH unit or more was established between homofermentative and heterofermentative LAB grown in HHD broth. Because of the pH values obtained with it (i.e., *Pediococcus pentosaceus* and *Leuconostoc mesenteroides* reached terminal pHs of 4.6 and 5.6, respectively, in HHD broth), bromscresol green (pK, 4.6) was chosen as the pH indicator. Incorporation of bromcresol green into the medium led to a color difference between broth containing homofermentative organisms and broth containing heterofermentative organisms. HHD broth inoculated with homofermentative LAB was green, while HHD broth containing heterofermentative LAB remained blue. In addition, the sedimented cells of the homofermentative species were blue to green, whereas those of the heterofermentative species were white.

TABLE 1. Formulation and preparation of HHD medium[a]

Component	Amt used	Manufacturer
Fructose	2.5 g/liter	Sigma Chemical Co.
KH_2PO_4	2.5 g/liter	Sigma Chemical Co.
Trypticase peptone	10.0 g/liter	BBL Microbiology Systems
Phytone peptone	1.5 g/liter	BBL Microbiology Systems
Casamino Acids	3.0 g/liter	Difco Laboratories
Yeast extract	1.0 g/liter	BBL Microbiology Systems
Tween 80	1.0 g/liter	Fisher Scientific Co.
Bromcresol green[b]	20.0 ml	Difco Laboratories
Agar (as desired)	20.0 g/liter	BBL Microbiology Systems

[a] Adjust pH to 7.0 ± 0.02. Autoclave at 15 lb/in² for 15 min.
[b] Stock solution is 0.1 g of bromcresol green in 30 ml of 0.01 N NaOH.

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TABLE 2. Differentiation on HHD medium of homofermentative and heterofermentative LAB in a 50:50 mixture

Microorganism mixture	% of organisms correctly differentiated[a] (mean ± SD)	
	Homofermentative	Heterofermentative
L. plantarum-Leuconostoc mesenteroides	97 ± 2.3	100
L. plantarum-L. brevis	97 ± 2.4	99 ± 1.6
P. pentosaceus-Leuconostoc mesenteroides	96 ± 1.4	99 ± 2.3
P. pentosaceus-L. brevis	97 ± 2.1	97 ± 2.2

[a] By presence or absence of gas in the Durham tube. Four replicate experiments were done for each identification.

The addition of agar allowed differentiation by colony color in a solid medium. Dilutions of *Lactobacillus brevis*, *Lactobacillus plantarum*, *P. pentosaceus*, or *Leuconostoc mesenteroides* were pour plated, overlaid with HHD agar, and incubated at 30°C. After a 3-day incubation, homofermentative colonies were blue to green, while heterofermentative colonies remained white. Also observed were differences in the color of the medium surrounding the colonies, with the medium ranging from blue to light green. This reaction was variable, depending on the density of the colonies and the length of incubation. It should be emphasized that differentiation of LAB type was based on the color of the sedimented cells or colonies and not on the color of the medium. To ensure consistency in colony classification, colonies should be identified against the black luminescent background of a colony counter.

Combinations of homofermentative and heterofermentative microorganisms (*L. plantarum-Leuconostoc mesenteroides*, *L. plantarum-L. brevis*, *P. pentosaceus-Leuconostoc mesenteroides*, and *P. pentosaceus-L. brevis*) in various proportions were pour plated and overlaid with HHD agar. After incubation at 30°C for 3 days, colonies were presumptively identified as either homofermentative or heterofermentative, depending on the color of the colonies from plates containing between 20 and 200 CFU. Colonies (20 to 60) were inoculated into tubes of MRS broth containing a Durham tube. Further identification of colony type was based on the presence or absence of gas formation in the Durham tube after overnight incubation at 30°C. In a 50:50 mixture of homofermentative and heterofermentative organisms, correct identification of the colony type could be made at least 96% of the time, regardless of the specific combination of organisms used (Table 2). In addition, mixtures containing any amount of homofermentative LAB could be correctly differentiated with at least 96% reliability (Table 3).

TABLE 3. Differentiation of mixed populations of homofermentative and heterofermentative LAB on HHB medium

% Homofermentative LAB in mixture	% of organisms correctly differentiated[a] (mean ± SD)	
	Homofermentative	Heterofermentative
100	100	
90	98 ± 1.4	97 ± 2.5
80	97 ± 2.0	97 ± 2.3
70	100	96 ± 1.9
60	100	96 ± 1.3
50	96 ± 2.4	99 ± 2.2
40	96 ± 2.4	97 ± 2.9
30	96 ± 3.0	97 ± 2.5
20	100	97 ± 2.5
10	100	100
0		100

[a] On the basis of the presence or absence of gas in the Durham tube. Identifications were done on four combinations of microorganisms in four replicate experiments.

By using HHD agar, it was possible to identify nine heterofermentative species (*Lactobacillus fermentum* ATCC 14931, *L. brevis* FBB 50, *Lactobacillus buchneri* ATCC 4005, *Lactobacillus cellobiosus* ATCC 11739, *Lactobacillus hilgardii* ATCC 8290, *Lactobacillus viridescens* ATCC 12706, and *Leuconostoc dextranicum* ATCC 19255 [all from the Food Fermentation Laboratory, Raleigh, N.C.]; *Leuconostoc mesenteroides* LC33 [from J. R. Stamer, Cornell University, Geneva, N.Y.]; and *Leuconostoc paramesenteroides* NRRL B-3471 [from the Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.]).

Twelve homofermentative species of LAB were also identified (*Streptococcus lactis* subsp. *diacetilactis* ATCC 13675, *Streptococcus faecium* LA16, *Streptococcus faecalis* LA18, *S. faecalis* subsp. *liquefaciens* LA20, *P. pentosaceus* FBB61, and *L. plantarum* WSO [all from the Food Fermentation Laboratory]; *Lactobacillus xylosus* NRRL B-449, *Lactobacillus casei* subsp. *pseudoplantarum* NRRL B-4560, *Lactobacillus salivarius* subsp. *salivarius* NRRL B-1949, *L. salivarius* subsp. *salicinius* NRRL B-1950, *Lactobacillus coryniformis* subsp. *torquens* NRRL B-4390, and *L. coryniformis* subsp. *coryniformis* NRRL B-4391 [all from the Northern Regional Research Center]. Pure cultures were plated and incubated as described above. After incubation, the species were classified as either homofermentative or heterofermentative, according to colony color.

High-pressure liquid chromatography analysis was done on the end products of the fermentation of HHD broth. Acids were analyzed with an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, Calif.). Sugars and alcohols were analyzed with a Sugar Pak-I column (Millipore Corp., Bedford, Mass.). A refractive index detector was used for both columns. High-pressure liquid chromatography analysis showed that homofermentative LAB produced 26 mM acid (lactic) and that heterofermentative LAB produced 14 mM acid (lactic plus acetic). In addition, mannitol was produced by heterofermentative LAB but not by homofermentative LAB. Fructose was not detected in HHD broth fermented by either homofermentative or heterofermentative LAB.

The difference in acid production and, consequently, the pH differential between homofermentative and heterofermentative LAB were the basis for differentiation of these LAB in HHD medium. These differences allowed the two groups to be distinguished in the agar medium containing a pH indicator. It is recommended that known cultures of lactic acid bacteria be included as comparative controls when unknown samples are tested. The majority of LAB can utilize fructose, but there are a few exceptions, such as *Lactobacillus sanfrancisco*, *Lactobacillus vaccinostercus*, and *Leuconostoc mesenteroides* subsp. *cremoris* (4, 7). Currently, we are attempting to make HHD selective for LAB only so that this medium can be used to differentiate naturally occurring LAB.

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