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Enumeration and identification of meat spoilage bacteria

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Enumeration and identification of meat spoilage bacteria

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SUMMARY

During prolonged storage of meat at chill temperatures, only a few types of organisms from the microbial flora originally present on meat are able to grow, and so appear in the spoilage flora. Therefore, spoilage flora of chilled meat usually contain only a limited number of bacterial types. Depending on the initial flora and the growth environment, only *Brochothrix thermosphacta*, *Shewanella (Alteromonas) putrefaciens*, a few species of *Pseudomonas, Acinetobacter, Moraxella, (Psychrobacter),* lactic acid bacteria, and some members of the family *Enterobacteriaceae* are significantly represented in most spoilage flora of chilled meats. These seven principal groups of spoilage bacteria can be readily differentiated from one another using the following seven properties: Gram-reaction, morphology, motility, the manner in which glucose is utilized, and the presence of catalase, oxidase and arginine dehydrolase/decarboxylase.

Spoilage bacteria can also be enumerated using selective and differential culture media, to suppress the growth of unwanted bacteria and assist the detection of the desired organisms. Those media have the disadvantage of commonly underestimating the bacterial population, but they can be used to conveniently identify changes in the numbers of specific groups of spoilage bacteria.

RESUMÉ

Lorsque la viande est entreposée à une température froide pour une période prolongée, peu d'espèces de la flore microbienne peuvent y croître et celles qui y réussissent font partie de la flore contaminante, responsable de la détérioration de la qualité de la viande. Dépendamment de la nature de la flore pionnière et du milieu de croissance, on retrouve les groupes suivants: *Brochothrix thermosphacta* , *Shewanella (Alteromonas) putrefaciens*, quelques espèces de *Pseudomonas*, *Acinetobacter*, *Moraxella*, *(Psychrobacter)*, des bactéries lactiques et quelques membres de la famille des *Entérobactériaceae*, sont aussi représentées de façon significative On peut différencier ces sept groupes principaux à l'aide de la réaction à gram, la morphologie ou motilité, la façon d'utiliser le glucose, la présence de catalase, d'oxydase et d'arginine déhydrolase/décarboxylase.

Ces bactéries peuvent également être dénombrées en utilisant des milieux de culture différentiels et sélectifs pour soit, supprimer la croissance des bactéries indésirables où pour aider à identifier celles qui pourraient être utilisées favorablement. Si ces milieux ont le désavantage de sous-estimer la population bactérienne, ils peuvent par contre être employés pour identifier de façon appropriée les changements dans le nombre de certains groupes de bactéries.

INTRODUCTION

Many species of microorganisms are able to grow on fresh meat. Their growth will eventually make the meat unacceptable for human consumption because of changes in the appearance, odour and flavour of the food (Dainty *et al.*, 1983; Gill, 1986). The changes caused by microbial activity will not occur if fresh meat is frozen to temperatures that are too low for microbial growth to occur. At chiller temperatures, -1.5° to +5°C, microbial growth is slowed, but is not prevented (McMeekin, 1981). However, spoilage is delayed by the slower rates of microbial growth. The rate of microbial growth can be further reduced by changing the atmosphere to which the bacteria are exposed. Both restricting the oxygen available to the bacteria and increasing the concentration of carbon dioxide tends to reduce the rate of growth of most spoilage bacteria. Chiller storage alone prevents the growth of mesophilic (cold intolerant) species, ensuring that only the few psychrotrophic (cold tolerant) organisms that were initially present on the meat are able to grow and form a spoilage flora. Consequently, floras developing on meat stored at chiller temperatures generally have a low species diversity (Ingram and Simonsen, 1980; Dainty and Mackey, 1992).

INITIAL FLORA ON MEAT

The bacteria found on skinned carcasses immediately after dressing are skin commensals, and soil and faecal organisms that were present on the hide. Most of those bacteria are Gram-positive mesophiles such as *Micrococcus, Staphylococcus*, and *Bacillus*, but a few Gram-negative psychrotrophic organisms will also be present. Unskinned pig carcasses that have been scalded, singed and polished will carry similar numbers of Gram-positive mesophiles and Gram-negative psychrotrophs, because the thermoduric (heat-tolerant) organisms associated with the skin that survive the scalding operation are augmented by psychrotrophic species derived from the dehairing and polishing equipment.

Growth of the Gram-positive mesophiles will be insignificant during carcass cooling, but substantial growth of the psychrotrophic species can occur when carcass cooling processes are poorly controlled.

When carcasses are broken down, bacteria are transferred from the contaminated carcass surfaces to the freshly cut meat surfaces. Additional mesophiles will be transferred to the meat from the hands of workers. Additional psychrotrophs will also be transferred from equipment when proper attention is not given to the cleaning of equipment.

The final hygienic condition of the meat when it leaves the fabrication line will reflect the overall hygiene of the dressing, cooling and fabrication processes (Egan and Roberts, 1987). When good hygienic practices are maintained in all

three processes, the average numbers of bacteria on beef and lamb are likely to be less than $10^3/cm^2$, with less than 5% of the flora being psychrotrophic, spoilage types. When hygiene practices are lacking in some respects, beef and lamb will carry bacteria at numbers greater than $10^3/cm^2$, with 10% or more of the flora being spoilage types. With pork, the initial contamination of the scalded carcasses will usually ensure that the average numbers of bacteria usually exceed $10^3/cm^2$, with about 50% of the flora being spoilage types.

SPOILAGE FLORA

The spoilage flora of chilled meat are usually dominated by the bacteria that grow most rapidly under the conditions experienced by the meat. The most rapidly growing species will fail to dominate only when heavy contamination with a slower growing competitor leaves insufficient time before spoilage for the growth rate advantage to be fully expressed. Fresh meat that is held in air at chill temperatures will develop a flora that is usually dominated by species of *Pseudomonas*, but with substantial fractions of the other strictly aerobic genera, *Acinetobacter* and *Moraxella*. Enterobacteria, and *Brochothrix thermosphacta* may also be present, if either formed a substantial fraction of the initial flora. The bacteria forming an aerobic flora will interact only when the maximum cell numbers are reached, at which time the bacteria apparently compete for the available oxygen. Aerobic spoilage bacteria grow by using soluble, low molecular weight compounds present at the meat surface. The bacteria that are primarily responsible for aerobic spoilage, the pseudomonads, use glucose preferentially. However, the bacteria eventually attain numbers so great that glucose cannot diffuse from the underlying meat to the surface sufficiently fast to satisfy the demands of the flora. The pseudomonads maintain their growth by switching to alternate substrates, notably the amino acids. When amino acids are used, malodorous compounds are generated as by-products. The putrid smells of those by-products indicate the onset of aerobic spoilage (Gill, 1986).

The rate of growth of the aerobic spoilage flora is slowed by high concentrations of carbon dioxide. However, the genera that dominate the spoilage flora of meat stored in air will still predomonate in aerobic atmospheres modified by the addition of carbon dioxide. The only means of precluding that dominance is to exclude oxygen from the atmosphere around the meat (Gill and Molin, 1991). The most common means of achieving anaerobic conditions is to vacuum package the meat in a film of low oxygen permeability. Biochemical activities of the muscle tissue then scavange any residual oxygen in the pack, and consume the small quantities of oxygen that permeate through the film. The spoilage flora of meat stored under anaerobic conditions is usually dominated by lactic acid bacteria of the genera *Lactobacillus, Leuconostoc* and *Carnobacterium*. Such lactobacilli will usually be the only bacteria distinguishable in anaerobic spoilage flora from muscle tissue of normal pH (5.5-5.8). A flora of that type will produce mild acid-dairy flavours in meat only some time after the flora has attained its maximum numbers. However, other types of

bacteria can grow on high pH (>5.8) muscle, and fat tissue that is not bathed in normal-pH exudate. Those bacteria are the facultative anaerobes of the family *Enterobacteriaceae*, and the species *Shewanella putrefaciens* and *Brochothrix thermosphacta*. Although the facultative anaerobes grow at slower rates than the lactobacilli, they produce by-products that are highly offensive. Thus, they will dominate the spoilage process even when they are only a minor fraction of the total bacterial population.

Meat flora may contain very different fractions of spoilage bacteria, and the spoilage activities of some types are disproportional to their relative numbers. Therefore, knowledge of the flora composition, as well as the total number, is required to understand and gain control over the spoilage process. Chill temperature spoilage flora are confined to a few bacterial types, so a short identification scheme that differentiates between the principal groups of spoilage organisms can be used for all but the most specialized investigations.

SPOILAGE MICROORGANISMS

Pseudomonas

Working definition: *Pseudomonas* species consist of Gram-negative, motile rods. They are strictly aerobic, catalase-positive, oxidase-positive and arginine-positive. They attack sugars by oxidation and do not produce gas.

Pseudomonads dominate the aerobic spoilage of chilled meat. The main species associated with raw meat are *P. fragi* and *P. fluorescens*. The pseudomonads preferentially use glucose. While using that substrate they do not produce malodorous compounds. However, when glucose is insufficient, they attack amino acids, forming sulphides, amines and esters that are organoleptically detected as putrid odours and flavours. Spoilage of normal pH (5.5-5.8) muscle tissue by *Pseudomonas* occurs when bacterial numbers exceed $10^8/cm^2$. However, muscle of dark, firm, dry (DFD) condition can be devoid of glucose and fat tissue is always glucose-deficient, unless it is bathed by muscle exudate. Spoilage of those glucose-deficient tissues occurs when bacterial numbers are only about $10^6/cm^2$. When bacterial numbers approach $10^9/cm^2$, slime becomes visible on the meat surface. When bacterial numbers are greater than $10^9/cm^2$ the aerobic bacteria stop growing, apparently because oxygen cannot diffuse fast enough into the bacterial slime to support further growth.

Acinetobacter/Moraxella (Psychrobacter)

Working definition: *Acinetobacter* species consist of Gram-negative, non-motile cocci or coccobacilli, which sometimes occur as pairs (diplococci). They are strictly aerobic, catalase-positive, oxidase-negative and arginine-negative. They attack sugars by oxidation or not at all.

Working definition: *Moraxella* species consist of Gram-negative, non-motile cocci or coccobacilli, which sometimes occur as pairs (diplococci). They are strictly aerobic, catalase-positive, oxidase-positive and arginine-negative. They do not attack sugars. It has been suggested that the family *Moraxella* should comprise the non-motile organisms previously assigned to the *P. fragi* group and *Psychrobacter immobilis*.

These strict aerobes commonly form a significant fraction of aerobic spoilage flora. Most do not utilize glucose, but degrade amino acids for all their growth on meat. However, they do not produce the highly offensive by-products that are associated with the degradation of amino acids by the pseudomonads. Therefore, bacteria of the *Acinetobacter/Moraxella* group have only a low spoilage potential

Enterobacteriaceae

Working difinition: The *Enterobacteriaceae* consist or Gram-negative, motile or non-motile rods. They are facultatively anaerobic, catalase-positive, oxidase-negative and arginine-negative or -positive. They ferment sugars, usually with gas production.

The family *Enterobacteriaceae* is comprised of a wide range of facultatively anaerobic organisms, that includes some pathogenic species. *Enterobacteriaceae* preferentially use glucose, then glucose-6-phosphate, before amino acids are attacked, with the release of malodorous by-products. Therefore, spoilage by organisms of this group is analogous to spoilage by the pseudomonads. They rarely contribute significantly to aerobic spoilage flora, because their aerobic growth rates are slow relative of those of the pseudomonads. However, they are important in the spoilage of vacuum-packed chilled meat, when the meat provides an environment of pH above 5.8. The genera of psychrotrophic enterobacteria commonly associated with chilled meat include *Serratia*, *Hafnia* and *Enterobacter*. Also, much raw meat is contaminated with small numbers of the mesophilic *Escherichia coli*.

Lactic acid bacteria

Working definition: This group of bacteria consists of Gram-positive, typically non-motile, non-sporing, non-acid-fast rods and cocci. They are facultatively anaerobic, catalase-negative, oxidase-negative and arginine-negative. They attack sugars fermentatively with the production of lactic acid.

These strictly fermentative organisms usually dominate the flora of meat stored anaerobically. Glucose, and perhaps the amino acid arginine, are the only substrates in meat that these organisms can use for growth. Maximum numbers are determined by the availability of those fermentable substrates, and do not exceed $10^8/cm^2$. The amino acids valine and leucine are also

metabolized by the lactobacilli. Although those substances do not support growth, they are degraded, with the slow production of volatile fatty acids that ultimately impart an acid-dairy flavour to the meat. That form of spoilage becomes apparent only well after the maximum numbers have been attained. The most prevalent genera in meat spoilage flora are *Lactobacillus, Leuconostoc* and *Carnobacterium*. The genus *Lactococcus* may appear in the initial flora, and can replace the predominant bacillary types in anaerobic spoilage flora at maximum numbers. The lactic acid bacteria can be homofermentative (producing lactic acid as the main product of glucose fermentation) or heterofermentative (producing a mixture of lactate, carbon dioxide and ethanol from glucose). The heterofermenters are the more important in the acid-dairy spoilage of meat.

Brochothrix thermosphacta

Working definition: *Brochothrix thermosphacta* is a Gram-positive, non-motile, non-sporing, non-acid-fast rod. It is facultatively anaerobic, catalase-positive, oxidase-negative and arginine-negative. It attacks sugars fermentatively.

This facultative anaerobe can occur in the flora of meat stored either in air or in vacuum packages. It is of greater importance in anaerobic than in aerobic spoilage flora, but only if the meat pH is above 5.8, as *B. thermosphacta* will not grow anaerobically at pH values below 5.8. It grows primarily by using glucose, producing acetic acid and acetoin under aerobic conditions, and lactic acid under anaerobic conditions. In addition, it can metabolize leucine and valine to produce isovaleric and isobutyric acids, which impart a strong aromatic odour to meat. Production of those odorous compounds gives this organism a high spoilage potential in both aerobic and anaerobic flora.

Shewanella putrefaciens

Working definition: *Shewanella (Alteromonas) putrefaciens* is a Gram-negative, motile rod. It is facultatively anaerobic, catalase-positive, oxidase-positive and arginine-negative. It attacks sugars oxidatively.

S. putrefaciens does not grow below pH 6.0, but on high-pH meat it can be the critical spoilage organism. Under aerobic conditions the spoilage behaviour of this organism is very similar to that of the pseudomonads, although it uses the amino acids cysteine and serine even when glucose is abundant. Under anaerobic conditions, the organism produces large amounts of hydrogen sulphide and organic sulphides from cysteine before the spoilage flora depletes the available glucose. It is therefore a potent spoilage organism and, if conditions allow its growth, it will promote early spoilage even when it is not numerically dominant in the spoilage microflora.

OTHER ORGANISMS ENCOUNTERED IN THE INITIAL FLORA

Micrococcus

Working definition: Species of *Micrococcus* are strictly aerobic, Gram-positive, non-motile cocci that are catalase-positive, oxidase-negative and arginine-negative.

These mesophilic organisms are major components of the normal flora of the mammalian skin, both human and animal. Consequently, they are usually found in substantial numbers in the initial flora on meat. However, as they are mesophiles they do not contribute to the spoilage flora.

Bacillus

Working definition: Species of *Bacillus* are aerobic, Gram-positive rods that form spores. Species may be motile or non-motile. All are catalase-positive, oxidase-negative and arginine-negative.

These organisms are common components of the flora on animals' skins, probably being derived from the animals' environment. Being mesophiles, they do not contribute to the spoilage flora of chilled meat.

Staphylococcus

Working definition: Staphylococci are facultatively anaerobic, Gram-positive cocci. They ferment glucose, and are catalase-positive, oxidase-negative and arginine-positive.

These organisms are part of the normal flora of mammalian skin and mucous membranes. Some species are opportunistic pathogens, and can occur in high numbers in skin lesions or the oro-nasal discharges from infected individuals. Enterotoxins produced during the growth of *S. aureus* and some other species in prepared foods are a major cause of food poisoning. The staphylococci characteristically divide in more than one plane, to appear under the microscope as tetrads and irregular clusters as well as single or paired cells. Being mesophiles, they do not contribute to the spoilage flora of chilled meat.

Vibrio

Working definition: Facultative anaerobic, motile, Gram-negative, straight or curved rods that ferment glucose, and are catalase-positive, oxidase-positive and arginine-positive.

This group of organisms includes psychrotrophic species that are important in the spoilage of some cured meats, to which they impart fishy-putrid

odours and flavours. Their occurance in the spoilage flora of cured meats derives from their advantaged growth in brines and salted products. They are found only occassionally in the initial flora on beef and lamb, and cannot be expected in the spoilage flora from those meats. However, pork is more commonly contaminated with vibrios, no doubt because the slaughtering of pigs and pork curing are often carried out within the same plant. When pork has been heavily contaminated with vibrios, those organisms may be found as a minor fraction of the spoilage flora.

SPOILAGE DEVELOPMENT

The conditions required by the principal groups of spoilage bacteria for growth on chilled meat are summarized in Table 1. The availability of oxygen and the pH of the meat are the most important factors that determine which types of bacteria will be represented in the final spoilage flora.

IDENTIFICATION OF SPOILAGE BACTERIA

Only five genera (*Pseudomonas*, *Acinetobacter*, *Moraxella*, *Brochothrix* and *Shewanella*), one family (*Enterobacteriaceae*) and the lactic acid bacteria are likely to be present in significant numbers in the flora developing on chilled meat. Those groups, and others likely to be found in the initial flora, can be differentiated using the properties of: Gram-reaction, morphology, presence of catalase, presence of oxidase, presence of arginine dehydrolase/decarboxylase, mode of glucose utilization, and motility (Table 2). That identification scheme is derived from the general scheme proposed by Cowan and Steel (Cowan, 1974). If bacteria other than those listed in Tables 2 are present in a flora, their identification will require the use of that or other general identification schemes, (Bergey, 1984; Blazevic and Ederes, 1975; McFaddin, 1980).

Table 1. Conditions for growth and the spoilage characteristics of the seven main groups of chilled-meat spoilage bacteria.

	Oxygen requirement	pH requirement	Spoilage potential	General remarks
Pseudomonas	Strict aerobe	None	High	Dominant in all aerobic spoilage floras
Acinetobacter	Strict aerobe	None	Low	Little significance for spoilage
Moraxella	Strict aerobe	None	Low	Little significance for spoilage
Enterobacteriaceae	Facultative anaerobe	No anaerobic growth below pH 5.8	High	Major spoilage organisms of vacuum-packaged, high-pH meat
Lactic acid bacteria	Facultative anaerobe	None	Low	Usually the dominant organisms of vacuum-packaged meat
Brochothrix thermosphacta	Facultative anaerobe	No anaerobic growth below pH 5.8	High	Major spoilage organism of some vacuum-packaged meats
Shewanella putrefaciens	Facultative anaerobe	No growth below pH 6.0	Very High	Major spoilage organism of some high-pH meat

Table 2. Differentiation of the principal groups of bacteria found on chilled meat.

	Gram reaction	Morphology	Catalase	Oxidase	Arginine metabolism	Glucose metabolism	Motility
Spoilage Flora							
Pseudomonas	-	bacilli	+	+	+	0/-	+
Acinetobacter	-	cocci/diplococci	+	-	-	-/0	-
Moraxella	-	cocci/diplococci	+	+	-	-	-
Enterobacteriaceae	-	short bacilli	+	-	-/+	F	+/-
Lactic acid bacteria	+	bacilli/cocci	-	-	+/-	F	-
Brochothrix	+	bacilli	+	-	-	F	-
Shewanella	-	bacilli	+	+	-	-/0	+
Initial Flora							
Micrococcus	+	cocci	+	-	-	0/-	-
Bacillus	+	bacilli	+	-	-	0	+/-
Staphylococcus	+	cocci	+	-	+	F	+
Vibrio[1]	-	bacilli	+	+	+	F	+

- negative reaction, + positive reaction, 0 oxidative, F fermentative.
[1]May appear in pork spoilage flora.

TESTS USED FOR DIFFERENTIATION OF SPOILAGE BACTERIA

GRAM REACTION

Preston and Morrell's modification of Gram's method is recommended, because it gives reliable results while allowing some variation in the duration of decolourization.

Solutions required:

Ammonium oxalate-crystal violet:

Crystal violet	20 g
Methanol	200 ml
Ammonium oxalate (1% w/v aqueous solution)	800 ml

Lugol's iodine:

Iodine	10 g
Potassium iodide	20 g
Distilled water	1000 ml

Dissolve the potassium iodide in a small volume of water, add and dissolve the iodine, then make up to 1000 ml.

Iodine-Methanol:

Iodine	10 g
Potassium iodide	6 g
Methanol	90 ml
Distilled water	10 ml

Iodine-acetone:

Iodine-methanol	35 ml
Acetone	965 ml

Ziehl-Neelsen's (strong) carbol fuchsin:

Basic fuchsin	10 g
Absolute ethanol	100 ml
Phenol solution, 5% in water	1000 ml

Dissolve the dye in the alcohol and add to the phenol solution.

Dilute carbol fuchsin:

Ziehl-Neelsen's carbol fuchsin	50 ml
Distilled water	950 ml

Procedure:

(1) Place a light suspension of cells on a slide, air-dry and heat-fix over a flame.

(2) Cover the slide with ammonium oxalate-crystal violet solution and leave for 30 seconds.
(3) Pour off the solution and wash the slide freely with Lugol's iodine. Cover with fresh Lugol's iodine solution and leave for 30 seconds.
(4) Pour off the Lugol's iodine solution and wash the slide freely with iodine-acetone solution. Cover with fresh iodine-acetone solution and leave for 30 seconds.
(5) Wash the slide thoroughly with water.
(6) Cover the slide with dilute carbol fuchsin solution and leave for 30 seconds.
(7) Wash the slide with water, blot off excess moisture and allow the slide to dry before it is examined.

It is essential that the whole slide is flooded with each solution in turn, and that the previous solution is thoroughly removed at each step.

MORPHOLOGY

The shape (morphology) of bacterial cells is determined by microscopic examination of the Gram-stained preparations.

Term	*Description*
Cocci	Spherical or nearly spherical cells.
Coccobacillus	Slightly elongate spherical cells.
Diplococci	Pairs of cocci, with the cells slightly elongated along the axis of the pair.
Bacilli	Straight, or slightly curved, rod-shaped cells.

CATALASE

This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide.

Pour 1 ml of 3% (10 vol) hydrogen peroxide solution (H_2O_2) over a 24-hr nutrient agar slope culture of the test organism, holding the tube in a slanting position.

Alternatively, pick a small amount of the culture to be tested from a nutrient agar slope or from a colony on a plate, using a clean glass rod. A suitable rod can be formed by sealing the tip of a Pasteur pipette. Place the sample on a clean microscope slide, they add a drop of 3% hydrogen peroxide solution.

The immediate production of gas bubbles from the surface of the culture material is a positive reaction.

Note. Do not pick material for this test with an inoculating loop, as platinum can catalyse the release of oxygen from H_2O_2.

OXIDASE

This test detects the presence of enzymes that catalyse the transport of electrons between electron donors in the bacterial cell and a redox dye, tetramethyl-p-phenylene-diamine. If an oxidase is present, the dye is reduced to a deep purple colour.

Reagent: 1% (w/v) aqueous solution of tetramethyl-p-phenylene-diamine dihydrochloride. This solution is unstable and must be stored in a refrigerator in a glass-stoppered bottle, protected from light. N.B. **The reagent should not be used if it has become deep blue**. Autoxidation can be retarded by the addition of ascorbic acid to a final concentration of 1% (w/v).

Soak a strip of filter paper with the reagent solution and immediately pick a sample of culture with a platinum loop, then rub the inoculated loop on the soaked paper. Development of an intense deep-purple colour within 60 seconds is a positive reaction If the purple colour developes after 60 seconds, or fails to develop, the reaction is negative. The test can also be carried out by adding the reagent to colonies on a Petri plate.

Note: The growth must be transferred to the test paper with a clean instrument made of platinum or glass, as traces or iron will catalyse the reaction to give a false positive result. If the colonies of a particular type are small, several colonies may have to be picked to obtain enough material to give a strong reaction. A positive reaction is often discernible within 10 seconds, but delayed reactions may require the full 60 seconds stipulated for the test.

ARGININE

This test detects the presence in bacteria of the arginine dehydrolase/decarboxylase enzyme system, which decarboxylates the amino acid arginine to give basic (alkaline) end products. Accumulation of those basic products causes the pH of the test medium to rise.

Arginine dehydrolase/decarboxylase media:

Basal medium:

Glucose	0.5	g
Sodium chloride	10	g
Trypticase (Tryptone)	10	g
Phenol red	0.05	g
Distilled water	1000	ml

The pH of the basal medium should be 7.2.

Test medium:

The test medium is prepared by adding 10 g of L(+) arginine monohydrochloride, or 20 g of LD (±) arginine monohydrochloride, to 1 litre of the basal medium. After addition of arginine, the pH must be readjusted to 7.2. Basal medium without the addition or arginine is used as a control.

Control and test media are dispensed into small screw-capped tubes (usually 13 x 100-mm tubes) to a depth of 15 to 20 mm. The filled tubes are sterilized at 121°C for 10 minutes. Tubes of both control and test media can be stored for up to 6 weeks at 4°C.

The control and test media are stab inoculated with a straight wire, then incubated at 25°C. The tubes are examined after 3, 5 and 7 days. At any of those times, a changed colour of the test medium, from orange to red, is a positive reaction.

GLUCOSE UTILIZATION

This test determines whether or not an organism utilizes glucose with the production of acid and, if glucose is so utilized, whether it is utilized oxidatively (O) or fermentatively (F). The O/F reaction of meat isolates is determined in Hugh and Leifson's medium using the single-tube procedure.

Hugh and Leifson's semi-solid medium:

Trypticase (Tryptone)	10	g
Yeast extract	1	g
Glucose	10	g
Bromocresol purple	0.04	g
Agar	3	g
Distilled water	1000	ml

Dissolve the ingredients in the distilled water and adjust the pH to 7.0.

The medium is dispensed, in 10-ml amounts, into screw-capped tubes (13 x 100-mm), then sterilized at 115°C for 20 minutes. Hugh and Leifson's medium will store for several weeks at 4°C. Before tubes of stored medium are used, they should be steamed for 10 minutes, then cooled rapidly in a bath of iced water. This procedure removes any oxygen that may have dissolved in the medium during storage. Failure to remove dissolved oxygen could allow strictly aerobic organisms to grow throughout the medium to produce the colour changes typical of glucose fermentation.

The test medium is stab inoculated with a straight wire, then incubated at 25°C. Tubes are examined after 3, 5 and 7 days. At any of those times, a change in the colour of the top third of the tube, from purple to yellow, is a positive oxidative reaction. The same colour change progressing from the botton of the tube is a positive fermentative reaction.

MOTILITY

The motility of bacteria can be determined from the O/F reaction tubes. Motile organisms show spreading growth from the initial line of the stab inoculation, whereas non-motile organisms are confined to the line of the stab. Motility can also be observed microscopically by moist mount, or by using commercially available motility test media.

SELECTIVE / DIFFERENTIAL MEDIA FOR SPOILAGE BACTERIA

INTRODUCTION

General purpose (enumeration) media such as Plate Count Agar (Standard Methods Agar) are nutritionally complete media formulated for the primary isolation and enumeration of bacteria from water and foods. As they are not selective, they support the luxuriant growth of most aerobic and facultatively anaerobic spoilage bacteria associated with meat.

The disadvantage of that type of medium is that is does not readily permit the differentiation of bacterial types, except on the uncertain basis of colonial morphology. Further biochemical characterization is necessary to properly identify the various types of colony that can be distinguished. Also, there are some organisms, such as lactic acid bacteria, which have complex nutritional requirements and grow poorly on the general purpose media.

It is possible, through the use of selective and differential agents and/or conditions, to suppress the growth of most groups of bacteria and so allow only one or a few specific groups to form colonies on the plate. A disadvantage with the use of selective cultural conditions is that often the growth of some strains of the targeted group is inhibited, with consequent underestimation of the numbers of the targeted group. However, a selective medium can be used to advantage when the objective is to detect the presence, or follow shifts in the numbers, of a spoilage organism or group of particular concern.

A medium is usually rendered selective by the incorporation of substances, such as antibiotics, that are inhibitory to many bacteria but to which the targeted organisms are relatively resistant. Appropriate choices of carbon and nitrogen sources, medium pH, and/or salt concentrations can also favour the growth of the targeted group. Further, incubation conditions favourable to the targeted group, but unfavourable to others, can be used.

It is usually not possible to formulate a medium that severely inhibits all competitors with the targeted group of organisms. However, colonies of the targeted group can be distinguished from others if a differentiating system is incorporated in the medium. A differentiating system may involve a pH and/or

specific chemical indicator and/or a substrate characteristically utilized by the targeted group. Then, a distinctive change in the appearance of colonies and/or the surrounding medium, because of the reaction with the differentiating system, will allow colonies of the targeted group to be discriminated.

In contrast to the use of selective/differential media, the cultivation of some spoilage bacteria requires the use of complex culture media which have been formulated to support the growth of specific organisms that are fastidious in their nutritional requirements. Such media have been termed elective.

A number of the media described herein are commercially available, making preparation under industrial conditions relatively simple. The recipes and description have been modified from "Pharmacopoeia of Culture Media for Food Microbiology" (Baird *et al.*, 1987; 1989) which is an excellent source of media formulations that are appropriate to meat microbiology.

TOTAL COUNTS: PLATE COUNT AGAR (PCA)

Description

A nutritionally rich culture medium initially developed for enumerating bacteria from dairy products (Marth, 1978). It is now accepted as a standard medium for enumerating total bacteria from food in general. The medium is commercially available, being designated by some manufacturers as Standard Methods Agar.

Composition

	(g/L)
Tryptone	5
Yeast Extract	2.5
Glucose	1
Agar	15

Preparation

Suspend all the ingredients in 1 L of water. Boil the suspension to dissolve the ingredients, then autoclave for 15 min at 121°C. Cool in a water bath to 47°C. Dispense 15 ml portions into Petri dishes.

Properties

The medium has a pH of 7.0 and is light amber in colour. Prepared plates can be stored for 2 weeks at 4°C.

Plate inoculation and colonial appearance

Spread a 0.1 ml portion of each suitable sample over the entire plate. Incubate the plates in air; at 35°C for 48 h, for total mesophiles; or 5°C for 10 d, for total psychrotrophs.

The pour plate method can also be used, with the incorporation of 1 ml sample portions into 15 ml of tempered agar.

As this medium is not selective, the sizes, morphologies and colours of the recovered colonies will vary.

Plates having 30-300 bacterial colonies are counted, and the Standard Plate Count (SPC) or Total Viable Count (TVC) is reported.

Total anaerobic counts

To estimate the total anaerobic population, plates are inoculated as for the aerobic count but are then incubated in an anaerobic jar or a disposable anaerobic system.

Pseudomonas: CEPHALORIDINE FUCIDIN CETRIMIDE AGAR (CFC)

Description

This medium was initially developed for the selective enumeration of pseudomonads from poultry (Mead and Adams, 1977). It has since been used extensively in the isolation and enumeration of pseudomonads from red meats and other foods. The antimicrobial agents, cephaloridine, fucidin and cetrimide suppress the growth of most meatborne organisms other than *Pseudomonas* species.

Composition

	(g/L)
Heart Infusion Agar	40
Cephaloridine	0.05
Sodium fusidate	0.01
Cetyltrimethyl ammonium bromide	0.01

Preparation

Suspend the heart infusion agar in 1 L of distilled water, then boil to dissolve the ingredients. Sterilize the basal medium by autoclaving for 15 min a 121°C. Cool the sterilized medium to 47°C in a water bath. Prepare the selective agents as 1% solutions in distilled water and sterilize each by

membrane filtration. Aseptically, add 5 ml of the cephaloridine, 1 ml of the fucidin and 1 ml of the cetrimide solution to the cooled basal medium. Mix the medium by swirling before pouring plates.

Properties

The medium has a pH of 7.4 and is straw coloured. Prepared plates can be stored for 1 month at 4°C.

Plate inoculation and colonial appearance

Incubate spread plates, at 25°C for 48 h. Pseudomonads appear as round, cream-coloured colonies. The identity of colonies can be confirmed by flooding the plates with oxidase reagent. The *Pseudomonas* colonies then develop a deep purple colour.

Enterobacteriaceae: VIOLET RED BILE GLUCOSE AGAR (VRBG)

Description

The medium is useful for performing total counts of *Enterobacteriaceae* (Mossel *et al.* 1978).

Composition

	(g/L)
Peptone	7.0
Yeast extract	3.0
Sodium chloride	5.0
Glucose	10.0
Bile salts	1.5
Crystal violet	0.002
Neutral red	0.03
Agar	15 g

This medium is commercially available.

Preparation

Suspend the ingredients in distilled water. Boil the suspension to dissolve the ingredients. Do not autoclave the medium. Cool to 47°C in a water bath.

Properties

This medium has a final pH of 7.4. It is a purple-violet colour. It must be prepared immediately before it is used.

Plate inoculation and colonial appearance

Add 10 ml of molten agar to 1.0 ml of the inoculum in a Petri dish. Swirl the dish to disperse the inoculum throughout the agar. Allow the basal layer to hardened, then overlay it with 5 ml of the same medium. Incubate in air; at 35°C for 18-24 h, for total enterics; or at 5°C for 10 d, for psychrotrophic enterobacteria. Enterobacteria appear as red-purple colonies that are surrounded by a red-purple halo.

LACTIC ACID BACTERIA: deMAN, ROGOSA AND SHARPE AGAR (MRS)

Description

MRS agar was developed for the cultivation of lactobacilli (de Man, Rogosa and Sharpe, 1960). The medium is not selective, but some selectivity can be gained by adjusting the pH to 5.7, by addition of 1 N HCl, and/or using anaerobic incubation conditions. Unfortunately, reducing the pH of the medium may restrict the growth of some meatborne lactics, such as *Carnobacterium*.

Composition

	(g/L)
Peptone	10
Beef extract	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan monoleate	1.0
Dipotassium phosphate	2.0
Magnesium sulphate.$7H_20$	0.2
Manganese sulphate.$7H_20$	0.05
Ammonium citrate	2.0
Sodium acetate	5.0
Agar	15.0

This medium is commercially available.

Preparation

Suspend the ingredients in 1 L of distilled water. Boil, to dissolve the ingredients, then autoclave the solution for 15 min at 121°C. Cool the sterilized solution to 47°C in a water bath, then dispense 15 ml portions into Petri dishes.

Properties

The medium has a final pH of 6.2, and is amber in colour. Prepared plates can be stored for 2 weeks at 4°C.

Plate Inoculation and Colonial Appearance

Incubate spread plates in an anaerobic jar, for 72 h at 25°C. Lactic acid bacteria appear as small, white or grey colonies. Colony diameters range from 0.5 to 2.0 mm. Colonies of typical lactic morphology can be confirmed by the catalase test, as lactics are catalase negative, while most likely contaminants are catalase positive.

Carnobacterium: CRESOL RED THALLIUM ACETATE SUCROSE AGAR (CTAS)

Description

MRS medium, particularly the acidified version, does not favour the growth of carnobacteria (*Lactobacillus divergens*), an important group of the meatborne lactic acid bacteria. The medium described is selective for *Carnobacterium*

Composition

	(g/L)
Peptone	10
Yeast extract	10
Sucrose	20
Tween 80	1
Sodium nitrate	15
Manganese sulphate. $7H_20$	4
Dipotassium hydrogen sulphate	2
Thallium acetate	1
Nalidixic acid	0.04
Cresol red	0.004
Triphenyltetrazolium chloride	0.01
Agar	15

Preparation

Suspend all the ingredients, except the triphenyltetrazolium chloride in 1 L of distilled water. Boil the suspension to dissolve the ingredients. Cool the solution in a water bath to 55°C, then adjust the pH to 9.1 using I N NaOH. Sterilize the solution by autoclaving, for 10 min. at 121°C, then again cool the solution to 55°C. Add triphenyltetrazolium chloride, as 10 ml of a 10%, filter-sterilized solution. Mix the completed medium by swirling before dispensing it into Petri dishes.

Properties

The prepared medium appears purple-red and contains precipitated material. The pH is 9.0. It can be stored for 1 week at 4°C.

Inoculation and colonial appearance

Spread plates are incubated in air, for 4 d at 25°C. *C. piscicola* produce bronze-metallic, yellow to pink colonies that are surrounded by yellow medium that is cleared of the precipitate. *C. divergens* produces pin-point colonies with a bronze-metallic shine. It may or may not produce a colour change in the medium.

Brochothrix thermosphacta: STREPTOMYCIN THALLOUS ACETATE ACITIDIONE AGAR (STAA)

Description

This medium was originally formulated for the selective isolation and enumeration of *Brochothrix thermosphacta* from meat (Gardner 1966). The combination of antimicrobial agents excludes most other meatborne bacteria.

Composition

	g/L
Peptone	20
Yeast extract	2
Glycerol	15
Dipotassium hydrogen phosphate	1
Magnesium sulphate.7 H_2O	1
Agar	13

Selective Agents

Streptomycin sulphate	0.5
Actidione (Cycloheximide)	0.05
Thallous acetate	0.05

Preparation

Prepare the basal medium by dispersing the non-selective ingredients in water. Boil the suspensions, to dissolve the ingredients, then autoclave the solution for 15 min at 121°C. Cool the sterile solution to 47°C in a water bath. Dissolve the selective agents in distilled water to give stock solutions, that each contain 50 mg/ml of the agent. Sterilize each stock solution by membrane filtration. Add 10 ml of the streptomycin sulphate solution and 1 ml each of the actidione (cycloheximide) and thallous acetate solutions to the cooled basal

medium. Mix the complete medium by swirling before dispensing it into Petri dishes.

Properties

The medium has a final pH of 7.0 and is a pale straw colour. Prepared plates can be stored for 1 week at 4°C and the filter-sterilized selective agents for 1 month at 4°C.

Plate inoculation and colonial appearance

Spread plates are incubated in air, at 25°C for 48 h. *B. thermosphacta* appear as white colonies 0.5 to 1 mm in diameter.

Brochothrix thermosphacta: STREPTOMYCIN SULPHATE INOSITOL NEUTRAL RED AGAR (SIN)

Description

This is a medium selective for *Brochothrix thermosphacta (*Schillinger and Lücke 1987). It allows the recovery and differentiation of *B. thermosphacta* in the presence of lactics, pseudomonads and enterics.

This medium is not widely used but it has the advantages over STAA of improving the recovery of *B. thermosphacta* and enabling detection and enumeration using Hydrophobic Grid Membrane Filtration.

Composition

	(g/L)
Blood agar base	40
Yeast extract	2
Dipotassium hydrogen phosphate	1
Magnesium sulphate.$7H_20$	0.8
Sodium carbonate	0.35
Myo-Inositol	10
Neutral red (0.3%)	10 ml

Preparation

Suspend all the ingredients in 1 L distilled water. Boil the suspension to dissolve the ingredients, then autoclave the medium for 15 min at 121°C. Cool the sterilized medium to 47°C in a water bath. Prepare a solution containing 50 mg/ml of streptomycin sulphate. Sterilize the solution by membrane filtration, and add 10 ml of the sterile solution to the cooled medium. Mix the completed medium by swirling before dispensing it into Petri dishes.

Properties

The medium has a pH of 7.0 and is pale red in colour. Prepared plates can be stored for 1 week at 4°C.

Plate inoculation and colonial appearance

Incubate spread plates in air, at 25°C for 48 h. *B. thermosphacta* appear as pink colonies, 0.5 to 1 mm in diameter.

Shewanella putrefaciens: PEPTONE IRON AGAR

Description

This differential medium was designed to detect hydrogen sulphide production by bacteria (Levin, 1968). The medium is useful for the isolation of *Shewanella putrefaciens,* when the condition of the meat suggests that the organism may be present as a substantial fraction of the spoilage flora.

Composition

	g/L
Peptone	15
Proteose peptone	5
Ferric ammonium citrate	0.5
Sodium glycerophosphate	1
Sodium thiosulphate	0.08
Agar	15

The medium is commercially available.

Preparation

Dissolve the ingredients in 1 L of water, then autoclave for 15 min at 121°C. Cool the solution to 47°C in a water bath.

Properties

This medium has a final pH of 6.7 and is light amber in colour.

Plate inoculation and colonial appearance

Add 10 ml of molten agar to 1.0 ml of the inoculum in a Petri dish. Swirl the dish to dispense the inoculum throughout the agar. Allow the basal layer to harden then overlay it with 5 ml of the same medium. Incubate in air at 25°C for

72 h. Colonies producing hydrogen sulphide will be grey-black and 1-2 mm in diameter. In some instances, blackening of the entire plate will occur.

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