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Technological Advancement in Methods of Microbiology in Milk Analysis- Part 1 (Direct methods)

This bulletin includes technical information based on latest developments on products, systems, techniques etc. reported in journals, companies' leaflets and books and based on studies and experience. The technical information in different issues is on different areas of plant operation. It is hoped that the information contained herein will be useful to readers.

The theme of information in this issue "**Technological Advancement in Methods of Microbiology in Milk Analysis- Part 1 (Direct Methods)"** It may be understood that the information given here is by no means complete.

In this issue:

INTRODUCTION

The importance of microbiology to the dairy industry has been demonstrated by frequent outbreaks of foodborne illness associated with consumption of milk and dairy products that had been contaminated with pathogenic organisms or toxins. Undesirable microorganisms constitute the primary hazard to safety, quality, and wholesomeness of milk and dairy foods. Consequently, increased emphasis is required to be placed on maintaining microbiological quality as well as analysis of milk and dairy products designed to evaluate the quality to ensure safety and regulatory compliance.

The need for effective HACCP programme for Total Quality management in the dairy industry and use of high speed production line has increased demand for microbiological surveillance of products, process, and environment have leading to rapid microbiological methods development and analysis. Several methods for rapid detection, isolation, enumeration, and characterization of microorganisms are being adapted by the dairy industry.

The existing conventional method by using for enumeration of total viable count is been used for several years. The operation of the conventional standard plate count method, although simple, is time consuming both in terms of operation and data collection. This method involves preparing the sample, dilution of sample, plating the sample through dilution with a specific agar, incubation and counting the colonies after pre-determined time. This method needs large no of consumables and requires the clean-up of reusable materials and re-sterilizing them for further use. Considering these

limitation of conventional methods several new approaches are highlighted in this document.

I. Micro-colony and single cell detection method

1. Flow Cytometry

The principle behind flow cytometry is quite simple: a suspension of cells is stained and forced through a capillary tube, which is illuminated in front of microscope objective. Every passing cell is registered by photo electronics attached to the microscope. Most microorganisms are optically too similar to resolve from each other or from debris; therefore, labeling with fluorescent dyes can be used to probe the viability (SYTO-9 in combination with propidium iodide) and metabolic state (e.g., ChemChrome (AES Chemunex)) of microorganisms. By using nonspecific and specific fluorochromes, as well as different wavelengths, and by measuring at different angles, it is possible to discriminate between bacteria in mixed populations.

For routine analyses of milk, water, beverage, or dairy industries flow cytometry is used. The examples of this method for practical use in the field is BactoScan (Foss Electric, Denmark), ChemFlow (Chemunex, France), Argus Flow Cytometer (Skatron, Norway).

2. Direct Epifluorescent Filter Technique (DEFT)

This method uses membrane filtration and epifluorescence microscopy, takes less than 30 min to complete is suitable for milk containing 5 x 10^3 to 5 x 10^8 bacteria per ml and does not suffer from many of the disadvantages of other microscopic methods.

Pre-treatment of the milk with proteolytic enzyme and surfactant at 50°C lyses somatic cells and makes fat globules sufficiently fluid to enable 2 ml of milk to be filtered routinely through a 25 mm diameter membrane fiter. Filtration concentrates and distributes the bacteria in a manner that makes counting easier and the techniques 100 times more sensitive than the Breed smear.

Method:

a. Whole milk, enzyme and surfactant are incubated at 50°C for 10 min in a sterile capped test tube.

b. A polycarbonate membrane filter (Nucleopore, 0.6-µm pore size) is mounted, shiny side uppermost, in a membrane filtration unit of a manifold connected to a vacuum line.

c. The filter is warmed by filtration of surfactant at 50°C before the treated milk is filtered. Surfactant at 50°C is used to rinse the test tube and filter tower after filtration of the treated milk. After filtration of the treated milk and rinse, the membrane is overlaid with stain.

d. After 2 min the vacuum is reapplied and the membrane is rinsed with buffer followed by isopropanol. The stained membrane is air dried and then mounted in non-fluorescent immersion oil on a slide beneath a cover slip.

e. The mounted membrane filter is then examined by means of an epifluorescence microscope suitable for use with acridine orange.

f. In the DEFT, because of the higher RNA content, metabolically active bacteria fluoresce orange whereas inactive bacteria fluoresce green.

g. Clumps of orange fluorescing bacteria are counted in microscopic fields of view taken at random.

Depending on the number of clumps per field, bacteria in 2-15 fields are counted. The DEFT count per millilitre is estimated from the mean clump count per field and the microscopic factor (MF).

The MF depends upon the amount of sample filtered through the given area of the membrane and the area of the microscopic field. The MF calculated as follows.

$$\mathbf{MF} = \frac{\text{Area of membrane through which sample is filtered } (mm^2)}{\text{Microscopic field area } (mm^2) \text{ X sample volume (ml)}}$$

II. Modifications of culture - based methods

1. SimPlate® (Bio Control System)

Detection and enumeration of microorganisms by the SimPlate® methods rely on a binary detection technology. It uses IDEXX's Multiple Enzyme TechnologyTM (METTM) to detect bacteria in food and in water. Visible colour changes occur as a result of bacterial enzyme reactions with substance present in the liquid culture medium.

a. The SimPlate system, (Bio-Control, Bellevue, WA) has 84 wells imprinted in a round plastic plate.

b. After the lid is removed, a diluted food sample (1ml) is dispensed onto the centre landing pad, and 10 ml of rehydrated nutrient liquid provided by the manufacturer is poured onto the landing pad.

c. The mixture (food sample and nutrient liquid) is distributed evenly into the wells by swirling the SimPlate in a gentle, circular motion. Excessive liquid is absorbed by a pad housed in the unit.

d. After 24 h of incubation at 35°C, the plate is placed under UV light. Positive fluorescent wells are counted, and the number is converted in the most probable number (MPN) table to determine the number of bacteria present in the SimPlate.

The method is simple to use with minimum amount of preparation. A 198-well unit is also available for samples with high counts. Using different media, the

unit can also make counts of total coliforms and *Escherichia coli* counts, yeast and mold counts, and even Campylobacter.

2. Petrifilms

Petrifilm plates are alternative to conventional agar plate methods for microbiological testing of food and beverages. This system can be classified as an improvement on traditional colony count methods. In this film with appropriate dry rehydratable culture medium systems containing nutrients, cold water soluble gelling agents and indicators embedded in a series of films in the unit to facilitate colony enumeration. The procedure of the method is as follows.

a. To obtain a viable cell count, the protective top layer is lifted, 1 ml of liquid sample is introduced to the center of the unit, and then the cover is replaced.

b. A plastic template is placed on the cover to make a uniform distribution of sample and round mold.

c. The rehydrated medium will support the growth of microorganisms after suitable incubation time and temperature.

d. The colonies are directly counted in the unit. Recently the company also introduced a Petrifilm

counter, the unit automatically count and record the viable cell count in the computer.

It is simple to use, is small in size and has a long shelf life over one year in cold storage. doesn't require preparing agar, and has results that are easy to read. Dry rehydratable films are marketed by 3M® for enumeration of aerobic count plate, Enterobacteriaceae (including Salmonella, Shigella, and Yersinia, coliform & *Escherichia coli,* yeast and mold, selective *E. coli* count plate, high-sensitivity coliform count plate, rapid coliform count plate, staph express count plate and environmental Listeria count plate. Enumeration by using Petrifilms got International recognitions, certificates and validations.

3. Compact Dry Plates

The Compact Dry plates also have a dedicated user-friendly small plastic dish format that contains sterilised dehydrated nutrients and differentiating components. Similar to the petrifilm, Compact Dry plates are also thin, light, and convenient to handle sample diffuses automatically and evenly in to the plates.

The presence of chromogenic substrates and redox indicators stain the grown colonies on the incubated plates with different colors, facilitating interpretation of the plates. The results are validated with the conventional methods.

Compact Dry Total counts, Coliform, *E coli*, and Yeast and molds have AOAC PTM (Performance Tested Methods) certificate, and Compact Total Count has also MicroVal certificate (European validation and certification alternative methods according to ISO 16140).

4. Hydrophobic Grid Membrane Filter (HGMF)

Hydrophobic grid membrane filter is a new approach to microbiological enumeration, it consists of a micro porous membrane filter of 0.45µm pore size on which a grid pattern has been imposed.

a. A conventional membrane filter is placed on the surface of an agar medium, water containing dissolved nutrients from the medium is carried by capillary action up through the filters pores to feed the microorganisms, which are retained on the top surface during filtration.

b. This produces a microscopic film of moisture on the top surface of the membrane filter through which motile organisms can travel.

c. The hydrophobic grid pattern of an HGMF interrupts this film of moisture, preventing motile organisms from travelling beyond the boundaries of grid squares in which they have landed.

d. Also, wherever the hydrophobic materials has been deposited on the filter, the pores are

blocked and water is prevented from bringing nutrients to any stray microorganisms, which might be pushed on to a grid line as colony increase in size and fills its square.

e. The analyst can then count the squares as individual colonies. Automatic instruments are also available to count these square colonies in seconds.

This method has been used to test a great variety of foods in the past several decades. This methods also approved by AOAC official method. **ISO-GRID/NEO-GRID** (Neogen, Lansing, MI) tests are based on the concept of hydrophobic grid membrane filtration.

a. The filter membrane is embossed with hydrophobic ink to produce a grid containing 1600 individual squares.

b. The ink's hydrophobic nature contains the growth of an organism isolated during the filtration process within the square of capture.

c. Samples are first diluted in a sterile buffer. The ISO-GRID and/or NEO-GRID filtration unit is then placed onto a manifold connected to a vacuum source, and a sterile filter membrane is placed in the ISO-GRID filtration unit (NEO-GRID already contains a sterile filter membrane).

d. Using the vacuum source, a portion of the diluted sample is filtered through the filter membrane.

e. The membrane is then transferred to an agar plate prepared with a medium formulated to promote/differentiate the growth of the target organism.

Following incubation, the plate's squares containing presumptively positive colonies are counted to yield a most probable number (MPN) result by using following formula.

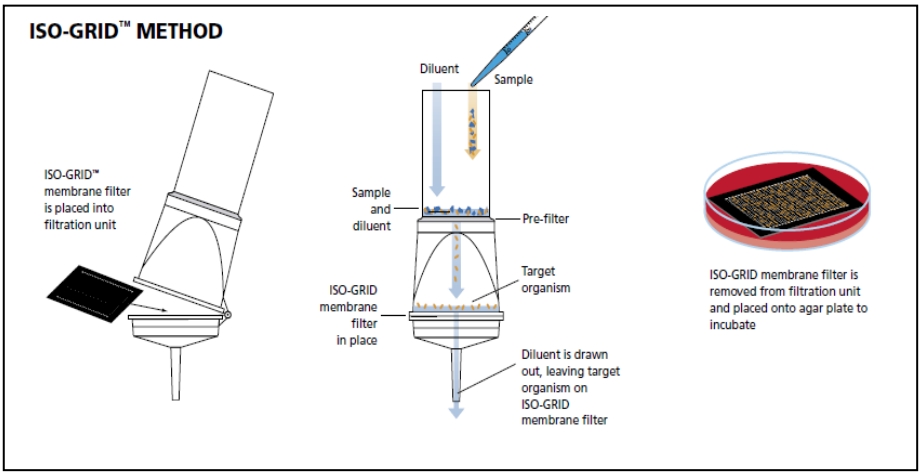
MPN= N X log c [(N/N-X)]

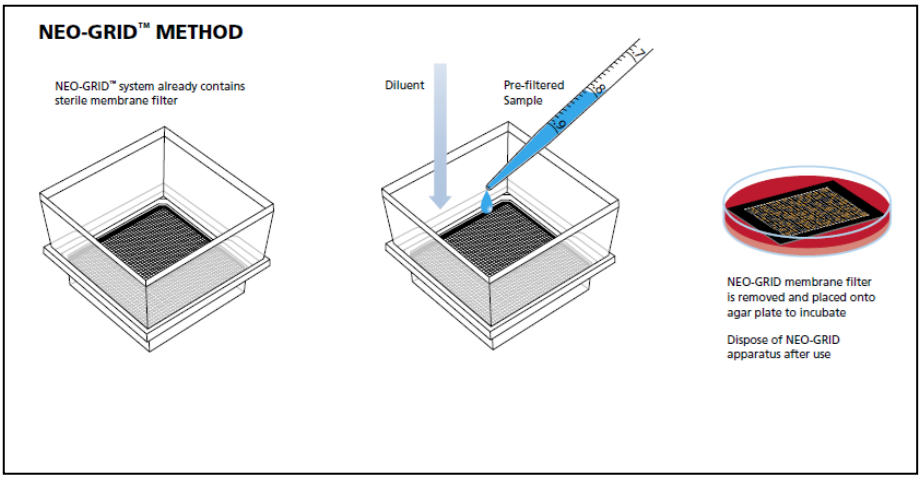
Where **N** = total number of grid squares on the filter; and
X =number of positive grid squares.

HGMF in Food Microbiology

The HGMF has formed the basis for a wide range of quantitative food microbiology applications, including total bacterial counts, coliform and Escherichia coli, fluorescent pseudomonads, lactic acid bacteria, yeasts and molds, Aeromonas, *E. coli O157:H7*, *Vibrio parahaemolyticus*, fecal streptococci, and *Staphylococcus aureus*. It also has been used for rapid detection of Salmonella and Yersinia, as well as for disinfectant efficacy tests.

Several of these applications have been validated and are recognized as Official Methods by AOAC International.





5. TEMPO® (Bio-Merieux)

The TEMPO® test is an 1st automated MPN enumeration method and consists of a vial of culture medium and a card, which are specific to the test. Dedicated equipment and software support the inoculation and reading of the cards.

a. The inoculated medium is transferred by the TEMPO® Filler into the card which contains 3 sets of 16 wells (small, medium, and large wells) with a 10 fold difference in volume for each set of wells.

b. The card is designed to simulate the MPN method but using 16 replicates instead of the usual 3 or 5, thus reducing the uncertainty of the method and enabling accurate quantification.

c. The card is then hermetically sealed in order to avoid any risk of contamination during subsequent handling. Target microorganisms multiply in the culture medium, resulting in a signal detected by the TEMPO® Reader (based on fluorescent pH indicator, β-glucuronidase activity, etc.).

d. The enumeration range is 10 to 49000 cfu/ml or 100 to 490000 cfu/ml, depending on the protocol.

TEMPO® automates food safety testing for total viable counts, coliform counts, *E. coli*, Enterobacteriaceae, lactic acid bacteria, *Staphylococcus aureus*, and yeasts/molds. Food pathogen detection testing for these organisms is important to a food quality laboratory for determining overall product hygiene and also as an indication of product spoilage. This method also got AOAC PTM status.

III. Immunological methods

1. Lateral Flow Devices

It's an immuno assay method, An LFD generally comprises a porous membrane, typically nitrocellulose, with an immobilized capture protein for the target analyte, forming a visible line in a viewing window, due to nanoparticles of gold or colored latex particles, after contact with the specified analyte.

For the test to be valid, a control line should form in a second viewing window. In most devices, an antibody is commonly used as capture protein, which specifically binds and captures a particular antigen if present in the sample. In most cases a sandwich assay is used. The test is fast (reading in terms of minutes) and simple both in use and in interpretation.

2. Enzyme-Linked Immunosorbent Assays and Enzyme-Linked Fluorescent Assays

ELISA is a biochemical technique that couples an immunoassay with an enzyme assay. In most of the

alternative methods, a sandwich ELISA is used. The sandwich ELISA comprises different steps.

a. Specific antibodies are affixed to the surface of the wells of a 96-well microtiter plate.

b. The sample, with an unknown amount of target antigen, is added and allowed to bind to the affixed antibodies. Unbound antigen is removed by a washing step.

c. In a second phase, antibodies targeting the antigen are added again to the wells. This step is followed by the addition of an enzyme-labelled secondary antibody.

d. This secondary antibody is allowed to bind to the previously added antibody.

e. Washing steps are included to remove non bound secondary antibodies.

f. In the final step, a substrate is added so that the enzyme can convert to a detectable signal. The ELISA detection itself only takes 2–3 hrs.

Today many ELISA tests are available as robotized automated systems not only to reduce the hands-on time but also to improve the reproducibility and standardization of each step of the assay.

3. Immunomagnetic Separation and Concentration

Super paramagnetic particles can be coated with antibodies, allowing specific capture and isolation of intact cells directly from a complex sample suspension

without the need for column immobilization or centrifugation. This method is now generically referred to as immunomagnetic separation and concentration.

a. In the ISO 16654 detection methods for *E. coli O157:H7* from foods, IMS is incorporated in order to pick up selectively the O157 serotype.

b. In Salmonella spp. detection methods, IMS is often integrated to replace the selective enrichment step, and thus approximately gaining 24hrs.

c. Monosized super paramagnetic polymer particles known as 'Dynabeads' are available commercially from Invitrogen-Dynal. Pathatrix, an automated system, is a patented recirculating immunomagnetic separation technology.

d. This technique enables to scale-up the application of IMS from the usual 1 ml to 400 ml volumes.

e. By recirculating the sample over a capture phase, comprised of immobilized antibody coated magnetic beads, the sensitivity of the capture is increased and thus accomplishes potential significant reduction of time to detection.

f. A high-volume wash also enables the efficient removal of the sample matrices, nonspecific microorganisms, and PCR inhibitors.

This technique is approved by AOAC international to perform as described.

IV. Nucleic acid – based methods

1. Fluorescent in situ Hybridization (FISH)

FISH is a cytogenetic technique used to detect the presence/ absence of specific rRNA sequences. FISH uses fluorescent probes that bind to only to target sequence with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe bound to the chromosomes. The choice to target RNA instead of DNA results in a more sensitive technique (higher copy numbers available) and the link to viability.

a. In the elaboration of FISH, microbial cells are treated with appropriate chemical fixatives and then hybridized under stringent conditions on a glass slide or in solution with oligonucleotide probes.

b. Generally, these probes are 15-25 nucleotides in length and are labelled covalently at the 50 end with a fluorescent dye.

c. After stringent washing to remove unbound probe, specifically stained cells are detected via epifluorescence microscopy.

d. The limit of detection is approximately 10^4 cfu/ml. After prior enrichment (usually overnight) to attain these levels of detection, results are available in 3 h, while the hands-on time required per analyse is only a few minutes.

FISH is commercially exploited by, for example, Vermicon, which has a detection kit for food pathogens

like Campylobacter, *E coli,* Enterobacter, Listeria, Salmonella, Staphylococcus and Yersinia.

2. Conventional, Real-time, and Multiplex Polymerase Chain Reaction (PCR)

The PCR technique is a three step cyclic in vitro procedure based on the ability of the DNA polymerase to copy a strand of DNA.

a. The region to be amplified is specified by the choice of primers. Primers are short oligonucleotides, usually 20-30 nucleotides in length, whose sequence matches the end of the region of interest. Amplification takes place over a number of cycles.

b. During each cycle, the double-stranded DNA template is denatured by heating to produce single strands. The reaction mixture is then cooled, allowing the primers to bind to the single strands.

c. This provides an active site for thermo-stable DNA polymerase, which synthesizes the complementary strand, producing again double-stranded DNA.

d. In subsequent cycles, primers will bind to both the original DNA and the newly synthesized DNA, resulting in an exponential increase in the number of copies.

e. The presence of even one copy of the template within the reaction mixture can be detected

within a couple of hours as about a million fold of copies are created.

f. The results of PCR are traditionally (conventional PCR) detected by agarose gel electrophoresis and staining.

g. This enables the amplified DNA to be visualized as bands differing in size. Specificity of the bands may be further identified by sequencing. PCR as such is taking only 30 to 90 min.

h. Indeed, PCR methods for detection of pathogens in foods recommended a 6 to 8 hrs up to 24 hrs prior enrichment step before execution of PCR, as only a small volume (1ml) is processed to extract DNA.

i. The inclusion of an internal control is recommended to highlight inhibition of the PCR reaction.

Real-time PCR is a rapid tool for screening of samples, still in case of positive PCR results, it should be tempted to confirm the positive result by means of the culture-based method.

3. Microarrays

Microarrays or gene chips provide a miniaturized system for the simultaneous analysis of hybridization of fluorescent labelled single-strand nucleotide chains to an array of oligonucleotide probes immobilized on a support such as glass or a synthetic membrane. PCR amplification is often used prior to hybridization to increase the sensitivity of detection.

DNA microarrays may be very useful for detecting multiple bacteria simultaneously on a single glass slide. The complexity of the food matrix is a major drawback of microarrays to be used as a detection method. Therefore, microarrays could be described as a tool for identification, genotyping, and pathotyping (detection of appropriate virulence factors) or characterization of bacterial isolates.

Since DNA arrays allow simultaneous measurements of thousands of interactions between mRNA-derived target molecules and genome-derived probes, they are rapidly producing enormous amounts of raw data never before encountered by biologists. The analysis of data on this scale is a major current challenge and therefore needs permanent attention to the issue of sample preparation and genetic material purification for successful and reliable analysis.

V. Combined methods

1. Biosensors

Biosensors are defined as analytical devices that combine bio specific recognition systems with physical or electrochemical signalling. It consists of two main components: a bioreceptor or biorecognition element, which recognizes the target analyte and a transducer,

for converting the recognition event into a measurable electrical signal.

a. A bioreceptor can be a tissue, microorganism, organelle, cell, enzyme, antibody, nucleic acid and biomimic etc. and the transduction may be optical, electrochemical, thermometric, piezoelectric, magnetic and micromechanical or combinations of one or more of the above techniques.

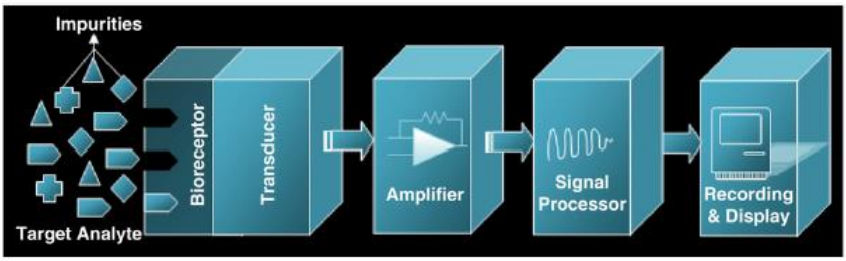
b. Biosensors for the detection of pathogens in the food industry consist of immobilized biologically active material, like enzymes, antibodies, antigens, or nucleic acids, in close proximity to a receiving transducer unit.

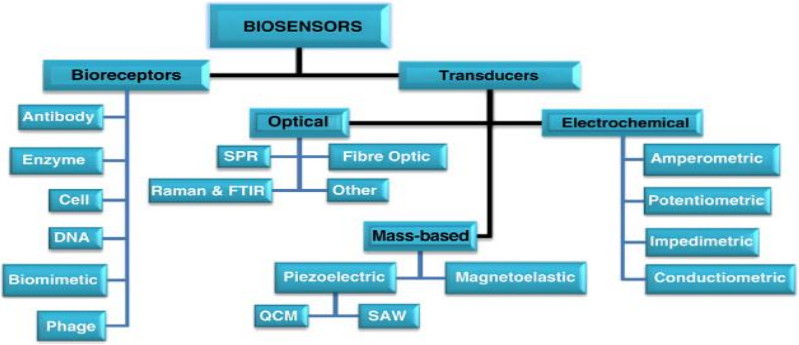
c. Target recognition results in the generation of an electrical, optical, or thermal signal that is proportional to the concentration of target molecules.

d. Examples of physical signals, which can report the presence of molecules, are fluorescence signals from dyes, electric fields from molecular charges, or mass changes or refractive index changes from the adsorption of molecules onto sensor surfaces.

e. Biosensors have the potential to shorten the time between sampling and results, but due to problems with long-term stability, reusability, and sterilizability, biosensors have so far been mostly used for detecting chemical substances.

Nevertheless, their future potential is enormous, since they can offer a very sensitive and accurate 'online' control system for food manufacturing processes. Below table depicts the working, types and various pathogens detection in food by using different models.





Methodological properties of selected rapid methods in food microbiology

Methods	Purpose			Detection limit cells (per ml or g)	Rapidity
	Quali.,	Quant.,	Char.,		
Direct Methods Epifluorescence microscopy					
DEFT	-	+	-	10^4	<1h
Ab-DEFT	+	+	+	10^3	<1h
MMCF	-	+	-	10^3	≥7h
Flow cytometry	+	+	-	10^4	<0.5h
Nucleic acid–based methods					
PCR	+	(+)	+	10^0 to direct in food 10^4	<2days
Dot Blot	+	(+)	+	10^4	<2days
Colony hybridization	+	+	+	10^1	<2days

qual., qualitative result (presence/absence); quant., quantitative result (enumeration); char., microbiological characterization; +, applicable; (+), applicability restricted; -, not applicable. a If special selective media are available

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