Microbiology Focus

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Fluka® Analytical

Clostridium perfringens: A Pathogen and Fecal Indicator

Clostridium perfringens is an indicator for fecal contamination and is also a pathogen. Water is one possible source for further contamination as the spores are quite resistant to heating and chlorination. A scanning electron microscope image of vegetative and spore forming cells from *C. perfringens*.

(source *C. perfringens* image: Copyright Dennis Kunkel Microscopy, Inc.)

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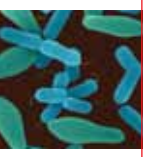

Fung Double Tube Method

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Four-hour rapid evaluation of fecal contamination of recreational water in seaside beaches by the Fung Double Tube Method.

Recreational waters such as those found at seaside beaches, rivers, lakes and ponds, are key areas for recreational use such as swimming, bathing, splashing, water games, etc. However, many of these waters may be contaminated with discharge, sometimes with fecal material from towns, cities, and small and large municipalities. These waters may contain fecal bacteria and other microbes which may pose a public health risk for swimmers, bathers and the general public who use these waters. Thus, it is important to monitor these waters regularly for the presence or absence of fecal contamination to protect the safety of the swimming public.

Conventional detection of fecal contamination in water takes one to two days. By the time the results are available, the quality of the water being tested may have changed substantially, making the data of very limited value.

In the past two years (2011–2013), the Water Research Group, a division of the Department of Public Health in Honolulu, Hawaii, under the direction of Dr. Roger Fujioka, has been testing newer and faster methods to ascertain potential fecal contamination of beach water. Using the unique Fung Double Tube Method, which can generate four-hour data from the time the water is applied to the system to the time results are read, allows a positive or negative result for fecal contamination. Currently, this is the fastest known method in the world for detection of fecal bacteria in water.

The heart of the test is as follows. *Clostridium perfringens*, an anaerobic bacterium which produces spores, is ubiquitous in fecal material (animal and human). Thus, the presence of *C. perfringens* in seawater would indicate a high probability of fecal contamination. The conventional method to detect the presence of *C. perfringens* in water takes 24 to 48 hours, making the results of very little value to warn the public of the potential danger of the water. Daniel Y. C. Fung, Ph.D., Professor of Food Science at Kansas State University, developed a Double Tube Method which can detect and enumerate *C. perfringens* in water in only four hours, making this the fastest test for fecal indicator organisms in the world. The reason for this success is that *C. perfringens* has the remarkably fast generation time of only 7.1 minutes at 41 °C, making it the fastest growing bacterium known to microbiologists at this time.

The group in Honolulu has been testing the Fung Double Tube System for about two years, and has found the method to be clean and very easy-to-use. It can indeed obtain four-hour results so that decisions can be made in four hours to open or close the beach for recreational use.

Did you know…

The production of *Clostridium perfringens* toxins starts under certain conditions?

Only one serotype out of five produces toxins, and the toxin production doesn't start until a concentration of 10^8 vegetative germs per gram of food or beverage is reached.

Figure 1: Keeping a pot simmering between 5–65 °C can provide good conditions for the growth of *C. perfringens*.

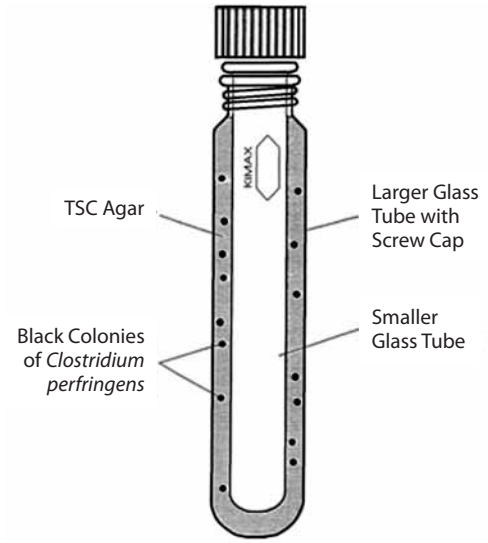

Figure 2: The Fung Double Tube System (for cultivation of anaerobes aerobically)

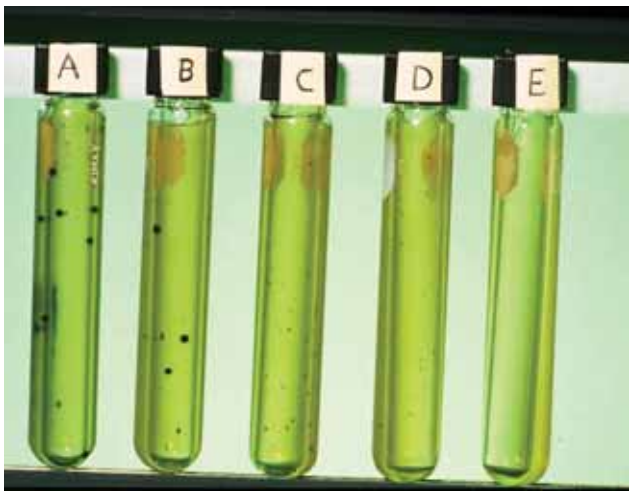

Figure 3: Each large tube contained 10 mL of the water sample selected for experimentation added into 10 mL of double strength (2X) melted sterile SFP agar. After rotating the larger tube with the sample and melted agar for 10 seconds to mix, a sterile inner tube is carefully inserted into the larger tube, thereby squeezing the liquid sample and SFP agar between the space of the two tubes, forming a thin film of sample and agar between the two tubes. The cap being tightly screwed, as well as a very tight, thin agar, makes the system very anaerobic, ideal for growth of *C. perfringens*. After about four hours of incubation at 41 °C, each single cell is visible as a black colony. *C. perfringens* is the fastest growing bacterium known; the generation time, under ideal conditions, is only 7.1 minutes.

The Fung Double Tube System involves two tubes—the smaller inside tube and a larger outer tube. Into the larger tube, 10 mL of liquefied Shahadi Ferguson Perfringens (SFP) agar (specially formulated to detect *C. perfringens*) was first placed and then sterilized. The medium is kept at 42 °C so that it will be in a liquid form. When a water sample is to be tested, 10 mL of the water (seawater, river water, etc.) will be carefully introduced into the large tube with the SFP agar. Then, the final step is to insert a smaller tube into the larger tube which contains both the SFP agar and the water sample. As the smaller tube is inserted into the large tube, the medium and sample will become sandwiched between the two tubes and form a thin film in the cavity. The material will be pushed up to the tip of the two tubes, then a screw cap will be applied, making this a very anaerobic system. In four hours, if there are *C. perfringens* in the water sample, tiny black colonies will form. The colonies will grow in size as time progresses, but at four hours, a researcher can start to ascertain if the water does or does not have *C. perfringens* and how many per mL of water.

If there are no black colonies found in the water, then it can be determined that the tested water is free of fecal material and safe to use for recreational purposes. If the number of colonies is 1–10, then the contamination level would be small and may be safe for swimming with caution. However, if the number is 10–100 colonies, the water is not safe for swimming, and the beach should be closed.

Rapid Detection of *Clostridium perfringens* by a New Chromogenic Media

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Selective chromogenic media for isolation and enumeration of *Clostridium perfringens* in water samples using membrane filtration.

CP *ChromoSelect* Agar is a selective chromogenic media for isolation and enumeration of *Clostridium perfringens* in water samples using membrane filtration. This new agar is more reliable and easier to handle than m-CP and TSC agars. The color does not diffuse in the agar and confirmation is not required since the green coloration is specific for *C. perfringens*. *C. perfringens* is an anaerobic, Gram-positive, spore-forming rod-shaped bacterium. It is widespread in the environment and is also found in the digestive systems of humans and domestic and feral animals. Perfringens poisoning, usually the result of ingesting under-cooked food, especially meat, is one of the most commonly reported foodborne illnesses. Early detection of *Clostridium* in food and water is important to control outbreaks. *C. perfringens* produces an extensive range of invasins and exotoxins. The enterotoxins cause the undesirable, mostly meat-associated food poisoning, and wound and surgical infections that lead to gas gangrene. *C. perfringens* plays a subsidiary role in water examination.[2] Clostridia are spore builders and are resistant to heating, chlorination and other stress factors. In contrast to vegetative cells like coliforms

Did you know…

***Clostridium perfringens* is a special anaerobe?**

It is a strictly anaerobic bacterium but is able to survive when exposed to oxygen for short periods of time. A complex adaptive response to reactive oxygen species was observed but not completely understood.

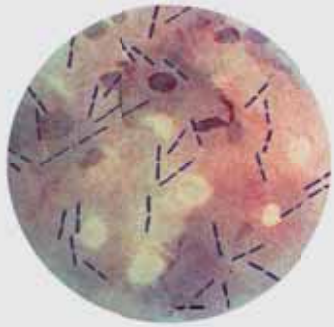
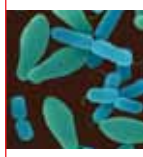
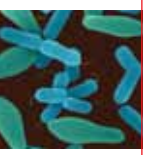


Figure 1: A photomicrography of a Gram-stained culture specimen from a patient with gas gangrene, showing numerous *Clostridium perfringens*. (Source: CDC 1979).





(*E. coli,* enterococci), which are less resistant, *C. perfringens* has the advantage of surviving longer.[3] Therefore, while fecal contamination is detected mostly by coliforms as an indicator, which could disappear after a processing step, *C. perfringens* remains present. The organism is not a hazard in water; rather, it is problematic when the water comes in contact with food. In consideration of the aforementioned facts, it is obvious that detection and identification of *C. perfringens* is an important step toward the control and eradication of this potent pathogen. Some characteristic enzymes of *C. perfringens* are: hemolysins (β-hemolysis), lecithinase, extracellular proteases, lipases (phospholipase-C), collagenase, hyaluronidase, saccharolytic, and enzymes to reduce sulphite to sulphide. These enzymes are also used as detection and differentiation targets. It is also notable that *C. perfringens* is a non-motile bacterium, and it is the most important of the sulphite reducing clostridia. *C. perfringens* normally grows at 44 °C, whereas some other clostridia are inhibited at this temperature. This property is used in ISO methods to give the medium more selectivity.[4]

Early detection of *Clostridium* in food is important to control outbreaks. To facilitate detection, we have introduced a new chromogenic media, CP *ChromoSelect* Agar, for enumeration and differentiation of *Clostridium* sp., in particular *Clostridium perfringens*, in aqueous samples. In the present study, three media types (mCP, TSCF and CP *ChromoSelect* Agar) were evaluated for recovery of *C. perfringens* in different surface water samples. Using a membrane filtration technique on 139 water samples, 131 samples (94.2%) were found to be positive for *C. perfringens* in at least one of the culture media. Green colored colonies on CP *ChromoSelect* Agar (**Figure 2**) were counted as presumptive *C. perfringens* isolates.

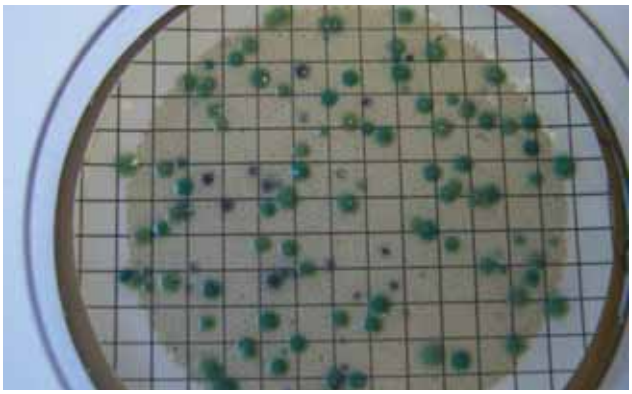


Figure 2: Drinking water sample cultured on CP *ChromoSelect* Agar. *C. perfringens* appears as distinct green colonies.

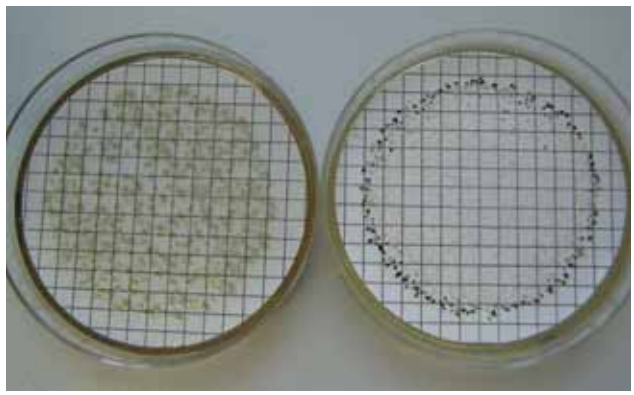


Figure 3: *C. perfringens* ATCC 10873 on CP *ChromoSelect* Agar (left) and TSC agar (right) (Note the false negatives on the TSC agar).

For detection of *C. perfringens*, m-CP and TSC agar have been recommended.[2,5] However, there are problems associated with each of these media. CP *ChromoSelect* Agar is more reliable and easier to handle than m-CP and TSC agars. The color does not diffuse in the agar and confirmation is not required since the green coloration is specific for *C. perfringens*. In addition, the recovery of *C. perfringens* was rejected by ISO in favor of methods based on TSC agar.[3,6] CP *ChromoSelect* Agar also eliminates the excessive and variable blackening of the peripheral colonies encountered with TSC agar, which makes colony counting at lower dilutions difficult, and leads to false positives. It is also more reliable at high bacteria counts, where the TSC agar can produce false negatives because of interference with the other enzymatic mechanisms from acid production and oxygen contact (**Figure 3**). TSC detects all sulphite-reducing clostridia, and not only *C. perfringens*.

Out of 483 green colonies on CP *ChromoSelect* Agar, 96.3% (465 strains, indole negative) were identified as *C. perfringens*, 15 strains (3.1%) were indole positive and were identified as *C. sordelli*, *C. bifermentans* or *C. tetani*. Only 3 strains (0.6 %) gave false positive results and were identified as *C. fallax*, *C. botulinum*, and *C. tertium* (**Table 1**). Variance analysis of the obtained data showed no statistically significant differences in the counts obtained between media used in this work (**Figure 5**). In general, the identification of typical and atypical colonies isolated from all media demonstrated that CP *ChromoSelect* Agar was the most useful medium for *C. perfringens* recovery in water samples.

CP *ChromoSelect* Agar avoids the disadvantages of m-CP agar, such as the presence of ammonia that prevents subculturing the *C. perfringens* colonies, the too-selective nature of m-CP agar and the evanescence of the red color of colonies after the addition of ammonia, which makes further confirmation impossible (**Figure 4**).

Strains	Indole Reaction	n
C. perfringens	–	465 (96.3%)
C. tertium	–	1 (0.2%)
C. botulinum	–	1 (0.2%)
C. fallax	–	1 (0.2%)
C. bifermentans	+	2 (0.4%)
C. sordelli	+	12 (2.5%)
C. tetani	+	1 (0.2%)

Table 1: All green colonies isolated from CP *ChromoSelect* Agar and identified with API system (n=483)

Besides its advantages over m-CP and TSC agars, CP *ChromoSelect* Agar is an ideal growth media. It contains only vegetable peptones and, together with yeast extract, it is an excellent source of nitrogen, carbon, amino acids and vitamin B complex. Sucrose acts as the fermentable carbohydrate, and reducing agents lower the redox potential of the media. Diverse salts provide the required ions for enzymatic reactions. Buffering agents stabilize the pH within the ideal growth range. Inhibitors Dcycloserine and polymyxin B give the medium its selectivity, while further selectivity is achieved by incubation under anaerobic conditions at 44 °C. Various promoters and substrates protect injured cells to improve recovery rate and enhance growth. The chromogenic enzyme substrates in the CP *ChromoSelect* Agar provide the differentiation, for *C. perfringens* in particular (**Table 2**). A negative indole reaction (Kovac's Reagent) is confirmatory for *C. perfringens*.

Organisms (ATCC)	Growth	Colony Appearance
Clostridium perfringens (13124)	+++	Green
Clostridium bifermentans (638)	+++*	Dark blue with violet halo
Clostridium sporogenes (8534)	–	–
Clostridium sordelli (9714)	++	Dark green with halo (change to red with Kovac's Reagent)
Enterococcus faecalis (29212)	++	Violet
Escherichia coli (25922)	–	–
Pseudomonas aeruginosa (27853)		Colorless
Staphylococcus aureus (25923)	–	–
Bacillus subtilis (6051)	–	–
Salmonella typhimurium (DSM 554)	++	Violet

Table 2: *Clostridium* sp. cultural characteristics in CP *ChromoSelect* Agar.
*Growth at 40 °C, but no growth at 44 °C

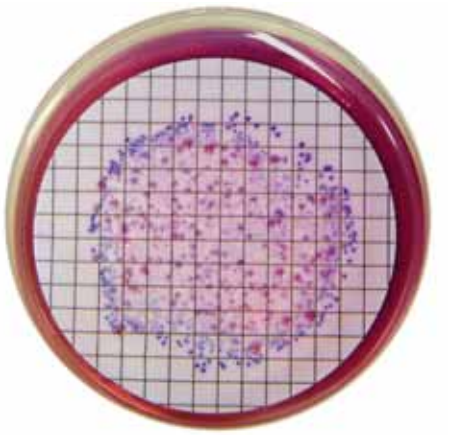


Figure 4: *C. perfringens* on m-CP Agar

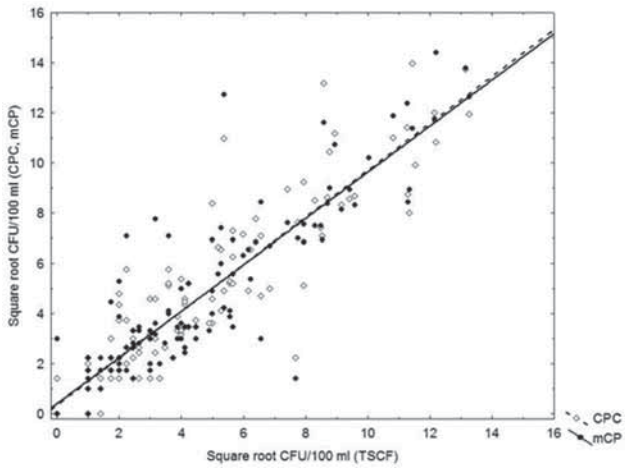


Figure 5: Comparison between TSCF agar and the m-CP and CPC (CP *ChromoSelect* Agar) media for enumerating strains of *C. perfringens* in water samples.

- CP *ChromoSelect* Agar was the most useful medium for *C. perfringens* recovery in water samples.
- CP *ChromoSelect* Agar is more reliable and easier to handle than m-CP and TSC agars. The color does not diffuse in the agar and confirmation is not required since the green coloration is specific for *C. perfringens*.

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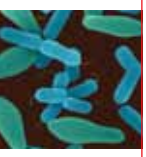

Sigma-Aldrich's range of media and supplements for *Clostridium perfringens* detection

Media	Description	Cat. No.
Nonselective Media		
AEA Sporulation Broth (Base), modified	For early sporulation of *C. perfringens* from foods.	17170
Nonselective Differential Media		
Gelatin Iron Agar	Used for detecting gelatin liquefaction and hydrogen sulphide production.	G0289
Lactose Gelatin Medium (Base)*	For the detection of lactose and gelatine metabolizing microorganisms (*C. perfringens*).	61348
Motility Nitrate Medium	Selective medium for motile nitrate-utilizing microorganisms (*Ps. aeruginosa, C. perfringens*).	14305
Selective Differential Media		
CP *ChromoSelect* Agar	A chromogenic agar for enumeration, detection and differentiation of *Clostridium* sp., in particular *C. perfringens*, in aqueous samples.	12398
m-CP Agar Base	Recommended by the Directive of the Council of the European Union 98/83/EC for isolation and enumeration of *C. perfringens* from water samples using membrane filtration technique.	75605
Perfringens Agar Base	Used for the presumptive identification and enumeration of *C. perfringens* from food.	39727
SPS Agar	Medium for detection, isolation and enumeration of *C. perfringens* and *C. botulinum* in food acc. to Angelotti et al. (1962).	85627
SPS Agar, modified	For the selective isolation and enumeration of *C. perfringens* from foods.	17231
TSC Agar* (Base for TSC Fluorescence Agar, SFP Agar, etc.)	For the isolation and enumeration of vegetative forms as well as spores from *C. perfringens* in food, clinical specimens and other material.	93745
TSN Agar*	Highly selective medium for the detection and enumeration of *C. perfringens* in food and other material.	93735

*not available in U.S.A.

Supplements	Cat. No.
Egg Yolk Emulsion	17148
m-CP Selective Supplement I	51962
m-CP Selective Supplement II	82265
Perfringens S.F.P. Selective Supplement	53436
Perfringens T.S.C. Supplement	P9352
TSC Agar Supplement	80548

Clostridium perfringens as Certified Reference Material

Evaluate the performance of your test with a certified microorganism standard.

Vitroids™ Test Strain	Origin	Strain No.	CFU	Cat. No.
Clostridium perfringens	NCTC	10240	30	RQC02351
			200	RQC02355
			500	RQC20106

Bifidobacteria in the Dairy Industry

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Bifidobacteria and the quality control of the dairy products they produce.

Bifidobacteria like *Bifidobacterium longum*, *infantis* and *brevi* are used for manufacturing dairy products. Bifidobacteria are Gram-positive, non-motile, rod-shaped and often branched obligate anaerobic bacteria. As probiotics, they have a positive affect on the immune system, control the intestinal pH and the gut microflora. Bifidobacteria produce bacteriocins and bacteriocin-like inhibitory compounds which hinder the growth of undesirable bacteria in the intestine. *Bifidobacterium* species were discovered by Tissier in 1889, and typically came to be associated with feces, especially those of breast-fed infants.[2]

B. longum is the best characterized species in the genus *Bifidobacterium*. It is able to utilize a broad range of substrates for energy, such as plant polymers, glycoproteins and glycoconjugates, as well as having specialized proteins for the catabolism of oligosaccharides like galactooligosaccharide (GOS) and fructooligosaccharide (FOS). GOS and FOS are prebiotics and are also often used as dietary supplements. A so-called bifidogenic growth stimulator (BGS) produced by *Propionibacterium freudenreichii* was observed in Swiss cheese, which improved the health of patients with ulcerative colitis. This was likely a prebiotic effect as well.[3]

Bifidobacteria also have a unique hexose metabolism called the bifid shunt. The key enzyme, fructose-6-phosphate phosphoketolase, is not found in any other Gram-positive intestinal bacteria and therefore provides an ideal target for a diagnostic test. The optimum growth temperature is 37–41 °C and they produce both lactic (L-isomer) and acetic acids from lactose. Sodium propionate is used to promote the growth of bifidobacteria. Cysteine helps in creating reduced conditions required for the growth of anaerobic bifidobacteria. *Bifidobacterium* species are known to be acid and bile tolerant. Other known antibiotics used for selective media are mupirocin, kanamycin B, polymyxin B, neomycin, paromomycin, gentamicin and nalidixic acid.

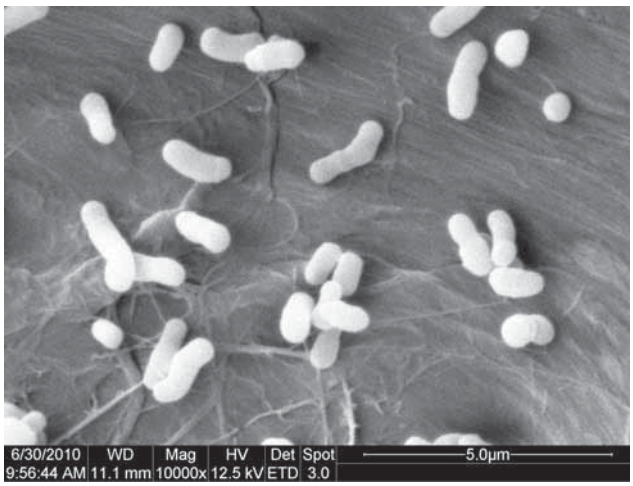

Figure 1: Scanning electron microscope image of *Bifidobacterium* (Source: Bao Qiuhua Inner Mongolia Agricultural University)

In an adult's intestine, only 3–6% of the fecal flora is composed of bifidobacteria, while in breast-fed infants, bifidobacteria can be up to 90%. With increasing age, the number of bifidobacteria decrease. Human milk contains many substances that stimulate the growth of bifidobacteria *in vitro*. It was observed that babies and adults with lower numbers of bifidobacteria have a higher risk for diarrhea and allergies. For this reason, *Bifidobacterium* are added as a probiotic supplement to infant formulas, drinks, yogurts and many other products.

Bifidobacteria Selective Media (BSM) with color reaction

Because of the wide use of bifidobacteria and a customer's request, Sigma-Aldrich has developed a Bifidobacteria Selective Media (BSM), available as an agar or a broth, for daily quality control. This medium allows for fast and easy quality control of yogurt made with bifidobacteria and can be used to control the count of bifidus bacteria. *Bifidobacterium* grows very well on this medium, while *Lactobacillus* and *Streptococcus* strains are inhibited. *Bifidobacterium* colonies grow within 24–48 hours (occasionally up to three days because of the highly selective conditions). The *Bifidobacterium* colonies are purple-brown and therefore easy to differentiate from other organisms.

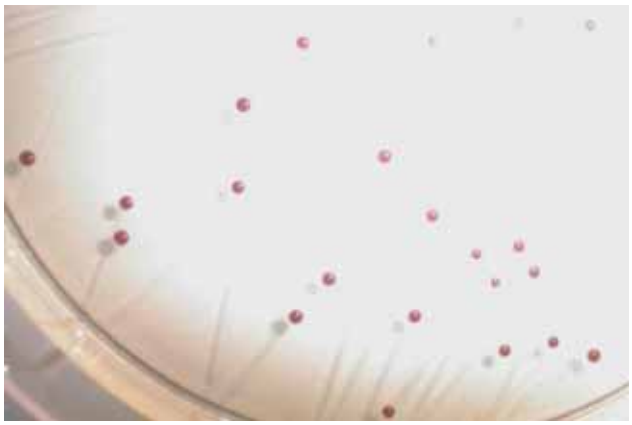

Figure 2: Yogurt sample cultured on BSM Agar. Bifidobacteria appear as purple-brown colonies.

In a Swiss governmental evaluation study for the enumeration of bifidobacteria in sour milk products, the traditional method was compared to Wilkins-Chalgren Agar with 100 mg/L mupirocine and BSM Agar. The traditional method gave significantly different results, while Wilkins-Chalgren Agar and BSM Agar showed similar results without any significant differences. The study remarked: "On the BSM Agar, the bifidobacteria forms purple-brown colonies which made the enumeration easy".[1]

Organisms (ATCC)	Growth	Colony Appearance
Bifidobacterium longum (15707)	+++	red-brown (maroon)
Bifidobacterium infantis (15697)	+++	red-brown (maroon)
Streptococcus thermophilus (14486)	–	–
Lactobacillus acidophilus (314)	–	–
Lactobacillus bulgaricus (11842)	–	–

Table 1: *Bifidobacterium sp.* cultural characteristics on BSM Agar

TOS-propionate agar

The TOS-propionate agar medium is recommended by ISO/IDF for enumeration of presumptive bifidobacteria by colony count technique from milk products with mixed flora. The medium is selective and other lactic acid bacteria are inhibited. In addition, the medium contains galactooligosaccharides, which is one of the most excellent bifidobacteria growth promoting substances. Lithium mupirocin is the antimicrobial supplement that inhibits the growth of most lactic acid bacteria commonly used in fermented and non-fermented dairy products.[4-6]

Name	Cat. No.
Bifido Selective Supplement B	90577-5VL
BSM Agar	88517-500G-F
BSM Broth	90273-500G-F
BSM Supplement	83055-5G-F
Lithium mupirocin Supplement	69732-10X5ML 69732-10X25ML
TOS-propionate Agar	43314-500G
Wilkins Chalgren Anaerobic Agar	W1761-500G

Table 2: Selective medium and supplements for *Bifidobacterium*

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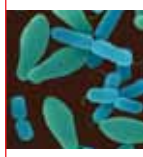

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