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Comparative Incidence of Coliform Bacteria and Enterococci in Undisturbed Soil[1]

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Within recent years there has been a renewed interest in the use of the enterococci as indices of fecal pollution. Suckling (1943) stated that these organisms were present in feces, sewage, and known polluted waters, whereas they could not be detected in unpolluted waters, virgin soils, and sites not exposed to human and animal life. It was also demonstrated that members of this group do not multiply outside of the animal body except in a medium such as milk. Litsky *et al.* (1953a) stated that the coliform group conventionally employed as indicators of pollution may consist of a large number of species of nonfecal origin. The members of the coliform group may persist in water and soil for long periods of time and therefore might not be indicative of recent pollution. It was also demonstrated that these organisms may multiply in a soil or water environment. Reliable differentiation between fecal and nonfecal strains is also a problem.

Prior to 1951, the greatest deterrent to the use of the enterococci as indices of pollution was the lack of a good presumptive medium for their detection. Because of this, relatively low numbers of these organisms were found in sewage and known polluted waters as compared to the coliform bacteria as demonstrated by Winslow and Nibecker (1903) and Lattanzi and Mood (1951).

With the development of dextrose azide broth (Difco)[3] as a presumptive medium and ethyl violet azide broth for confirmation, the detection of the enterococci was simplified. Using this method more enterococci were detected in polluted water and sewage than by any prior method as demonstrated by Mallmann and Seligmann (1950), Mallmann and Litsky (1951), and Litsky (1953b).

The effectiveness of the above two media for the detection and enumeration of the enterococci suggested that the presence of these bacteria in undisturbed[4] soils should be revaluated in an attempt to establish whether or not this group could be found outside the animal body in nature. As a means of comparison, the coliform incidence was also determined. The results of such an investigation would add another link to the

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[4] Soil sites undisturbed by cultivation according to records for the past 60 years.

chain of evidence as to the advisability for the promotion of the enterococci as indicator of recent fecal pollution.

Experimental Procedure

Three hundred and sixty-nine samples of soil were taken from areas adjacent to four Western Massachusetts water supply reservoirs. Past fecal contamination was either nonexistent or very remote due to the sanitary control of these water sheds. A record search indicated that manure fertilizing had not taken place in the test areas in the past 60 years. The present study extended over the winter, spring, and summer months of 1956.

Particular attention was paid to the nature of the terrain during the sampling process. Each area was studied for elevation and drainage by recourse to relief maps and personal observation. Each sampling site selected was on high ground and therefore had slight possibility of chance pollution as a result of contact with the water table or via surface or subsurface drainage.

Soil samples were taken from a depth of 1 to 3 in. below the surface of the ground using a conventional spade shovel and a portion was transferred in sterile, wide mouth, Bakelite covered, glass jars. The time elapsing between taking the first and last samples and actual testing on a given day was never more than 3 hr. Upon arrival at the laboratory, 10-g samples of soil were added in parallel into the respective presumptive media for the qualitative determinations of both the coliform group and the enterococci. These were then vigorously shaken in an automatic shaking machine for 3 min to insure a high surface to volume ratio between soil and medium which would yield a more sensitive qualitative determination, especially if the specific organisms were present in relative low densities.

Coliform Determination

Ten g of soil were added to 90 ml of lactose broth and shaken by the procedure mentioned above. The flasks were then incubated at 37 C for 48 hr. As confirmation of the presence of coliform bacteria, tubes of brilliant green bile broth (BGB) were seeded from the presumptive lactose broth flask. As a second confirmation test, Levine eosin methylene blue agar (EMB) streak plates were used. These were incubated for 48 and 24 hr, respectively, at 37 C. A positive confirmation test for coliform was recorded on the basis of detection of gas in the BGB broth and/or the formation of colonies on EMB agar which, upon subsequent inoculation and incubation, fermented lactose broth with the formation of gas (*Standard Methods for the Examination of Water, Sewage and Industrial Wastes*, 1955).

Enterococci Determination

The same basic enrichment procedure used for the coliform determination was used for the qualitative determination of the enterococci. Dextrose azide (Difco) was used as the enrichment medium and incubation was carried out for 48 hr at 37 C, after which time one drop of the agitated culture was transferred into ethyl violet azide broth (EVA) (Difco) for confirmation of enterococci. The presence of enterococci was indicated by turbidity and the formation of a purple "button" at the bottom of the confirmatory medium. Random positive tubes were examined microscopically for the presence of short chained, gram positive cocci. In addition, the tubes that showed turbidity but no purple "button" were further streaked on tryptone glucose extract agar (Difco) and incubated at 37 C for 48 hr. Isolated colonies were then picked at random and tested for the ability to grow at 45 C and in a 6.5 per cent sodium chloride tryptose broth. Growth in both environments, coupled with microscopic examination, was considered as complete evidence for the presence of enterococci.

Results

Typical colonies of *Escherichia coli* from EMB streak plates were isolated in only 4 of the 369 soil samples tested, whereas atypical coliform types were observed throughout this study. It was also observed that there was little correlation between BGB and lactose broth when these were used as the confirmative and completed test respectively. Seventy-six samples gave evidence of gas formation in BGB broth and none in the lactose broth of the completed test. Thirty samples gave gas formation in lactose broth of the completed test but none in BGB broth. Thirty-five samples gave atypical coliform reactions on EMB agar but no presence of gas in either BGB broth or lactose broth. Table 1 represents a tabulation of the frequency incidence and source of coliform bacteria as judged by the two qualitative procedures used.

Enterococci were found in 8 of the total of 369 soil samples examined (2.2 per cent). With one exception, the presence of enterococci in a given soil sample was associated with the presence of strong gas forming coliform organisms. Of the 8 samples containing enterococci, 2 showed the presence of *E. coli*. The results are summarized in table 2.

In determining the incidence of the coliform group from the above data, it was arbitrarily concluded that growth on EMB and gas formation in BGB broth and/or lactose broth of the completed test indicated the presence of the coliform bacteria. Using the above scheme, it was calculated that 99 samples or 26.8 per cent contained no coliform; 5 samples or 1.4 per cent contained *E. coli*; 265 samples or 71.8 per cent con-

tained other coliforms. Of the 270 samples which were positive for the coliform group, 23.0 per cent showed weak lactose fermenters.

Discussion

The presence of coliform bacteria in undisturbed soil appears to be indisputable and in confirmation of the results of previous investigators. Realizing that there are significant Chi-square results for the qualitative presence of coliform bacteria from one sampling area to another (Griffin and Stuart, 1940), the results of the present study very closely approximate the results obtained by Randall (1956). In that study samples of soil were collected from many different sources and were roughly grouped as follows:

Group I. Decaying garden vegetation mixed with soil.

Group II. Garden soil apparently free from manure.

Group III. Woodland soils.

Group IV. Soil from ungrazed grassy sites.

Group V. Soil from special grassy sites. These 98 samples were taken from the protected grassy slopes beside enclosed reservoirs.

The chances of pollution were thought by inspection to vary from group to group being greatest in group I and descending in order to group V. In general, Randall found as chances of pollution decreased the number of coliform intermediates increased significantly, whereas the *E. coli* incidence decreased very markedly. *Aerobacter aerogenes* showed no logical increase or decrease in incidence as did the number of samples which showed no presence of coliforms. Since Randall's group V approximates the type of soil examined in this study, it was of interest to compare the qualitative results of the present study with group V (table 3).

The significance of table 3 appears to lie in the indication that the sampling techniques used in the present study did indeed represent, for the most part, unpolluted or remotely polluted soil, as judged by the remarkable "fit" of the relative numbers of coliform intermediates, *E. coli*, and the number of samples which contained no coliforms. The writers realize, however, that differentiation of the coliform bacteria in the present study was obtained only in a rough measure by recourse to an EMB agar plate.

This investigation has revealed that the coliform group of organisms are indeed found in soil which is apparently undisturbed soil; however, *E. coli* and the enterococci are not found extensively. Logically, there appears to be a close relationship between the presence of *E. coli* and the enterococci group. Randall (1956) in determining the presence of the enterococci in unpolluted or very remotely polluted soil found that, of the 33 samples examined, 8 contained *Streptococcus faecalis*, and from 6 of these *E. coli* was also found. In the present study, the same fundamental relationship was also found. The presence of both in a given soil sample could be considered to be indicative of chance pollution of the sampling area by bird or animal droppings, or surface or subsurface drainage. From outward appearance, it seems that the enterococci and *E. coli* show equal results for the determination of the presence or absence of fecal pollution. However, when the factors of expedience and economy of materials are considered, the recommendation for using the enterococci group as the preferred indices of pollution seems justified.

TABLE 1

Tabulation of source and frequency incidence of coliform bacteria as judged by qualitative procedures A and B

Procedure A Brilliant Green Bile Broth	Procedure B		Source				
	Levine eosin methylene blue agar	Lactose	Amherst A	Amherst B	Springfield	Quabbin	Total
+	Typ*	+	2	0	3	0	5
+	Atyp	+	51	18	70	20	159
+	Atyp	−	26	12	37	1	76
−	Atyp	+	10	5	15	0	30
−	Atyp	−	14	7	14	0	35
+	Neg	−	0	0	1	3	4
−	Neg	+	1	0	0	0	1
−	Neg	−	10	4	22	23	59
Total...			114	46	162	47	369

* Typ, typical; Atyp, atypical; Neg, negative.

TABLE 2

Incidence of enterococci tabulated according to source and parallel occurrence of coliform bacteria

Sample No.	Source	Enterocci		Coliform	
		Ethyl violet azide broth	Brilliant green bile	Levine eosin methylene blue agar	Lactose
57	Amherst A	+	−	Neg*	−
64	Amherst A	+	+	Typ	+
75	Amherst A	+	+	Atyp	+
79	Amherst A	+	+	Atyp	+
90	Amherst A	+	+	Typ	+
162	Springfield	+	+	Atyp	+
170	Springfield	+	+	Atyp	+
223	Springfield	+	+	Atyp	+

* Typ, typical; Atyp, atypical; Neg, negative

TABLE 3

Comparisons of Randall's qualitative determination of coliform bacteria with that of the present study

	No. of Samples	No. Coliforms	*Escherichia coli*	Other Coliforms
		%	%	%
Randall's group V.....	98	23.4	10.2	66.4
Present study.....	369	26.8	1.4	71.8

SUMMARY

A study has been made to revaluate to what extent the coliform group and the enterococci are present in soil, which is apparently undisturbed soil, using modern techniques for their isolation and identification.

Three hundred and sixty-nine soil samples were taken from areas adjacent to four Western Massachusetts water supply reservoirs in which past fecal contamination was either nonexistent or very remote due to the sanitary control of the watersheds.

Two hundred and seventy samples (73.4 per cent) contained coliform bacteria, as indicated by the presence of gas in lactose broth seeded from eosin methylene blue agar plates, and/or presence of gas in brilliant green bile broth. Five samples (1.4 per cent) yielded typical *Escherichia coli* colonies on eosin methylene blue agar. Enterococci were found in 8 (2.2 per cent) of the 369 soil samples examined.

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Nutrition and the Development of Mushroom Flavor in *Agaricus campestris* Mycelium

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Only two varieties of *Agaricus campestris,* the common edible mushroom, are grown commercially in the United States. These are distinguished by their white and light brown color. In contrast, there are at least 75 varieties of mushrooms available or marketed in Europe.

The art of cultivating muchrooms was a carefully guarded secret dating back to the 17th century. It was not until the beginning of the 20th century that the first scientific approach to mushroom cultivation appeared in the literature of this hemisphere. Duggar (1905) successfully grew *A. campestris* on a variety of substances such as casein and peptone for nitrogen sources, and cellulose as a carbon source.

Waksman and McGrath (1931) studied the chemical changes occurring during composting of horse manure for mushroom culture and the changes resulting from the growth of the mushroom mycelium on this compost.

A number of investigators have observed the growth characteristics of *A. campestris* on so-called synthetic compost. Hein (1930) developed a straw compost and Szuecs (1931) a peat-molasses compost upon which mushrooms could grow.

Metabolic requirements of *A. campestris* were established in detail by Humfeld (1948, 1950–1951), and Humfeld and Sugihara (1949, 1952), who were the first to achieve growth of the organism under submerged, aerated, and agitated conditions in a chemically defined medium. With glucose as the carbohydrate source and urea as the only nitrogen source, they determined that the presence of K, Mg, Ca, and trace amounts of Fe, Mn, Zn, and Cu were essential for optimum growth as well as the development of the typical or true mushroom flavor in the mycelium. The optimum pH for mycelial growth was established at 4.5, and Szuecs (1958) obtained good submerged growth and flavor by culturing *A. campestris* and other species in a yeast extract-corn syrup medium.

Block *et al.* (1953) used a similar system for the sub-