DOMINION OF CANADA
DEPARTMENT OF AGRICULTURE
DOMINION EXPERIMENTAL FARMS

# DIVISION OF BACTERIOLOGY

## PROGRESS REPORT OF THE DOMINION AGRICULTURAL BACTERIOLOGIST

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FOR THE YEARS 1931, 1932 AND 1933

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# DIVISION OF BACTERIOLOGY

PROGRESS REPORT FOR THE YEARS 1931-32-33

## DAIRY BACTERIOLOGY

### Studies on Chlorine Sterilization

The difficulties encountered in obtaining adequate sterilization of dairy utensils and equipment with hot water have led to the use of chlorine solutions for this purpose. Studies have been carried out by the Division along the following lines:—

(*a*) Relative germicidal efficiency of various hypochlorite and chloramine-T products.
(*b*) Effectiveness of hypochlorite solutions against organisms causing ropy and bitter milk.
(*c*) Relation between alkalinity, germicidal speed and corrosiveness of hypochlorites.
(*d*) Chlorine sterilizing solutions as preservatives in milk and cream.
(*e*) Economy and practicability under farm conditions.
(*f*) Testing of methods for evaluating the germicidal potency of hypochlorites, and development of new methods.

In a previous report[1] investigations were described concerning the germicidal action of various commercial hypochlorite and chloramine-T products, the applicability of chlorine sterilization under farm conditions, and the preservative effect of chlorine compounds. During 1931 attention was given to the question of alkalinity as it affected germicidal speed and corrosiveness.

Previous studies had shown that strongly alkaline products like Diversol, while much less corrosive, were decidedly slower in germicidal action than the mildly alkaline products such as B-K and HTH. Consequently an attempt was made to develop a solution which would avoid as much as possible the disadvantages of both these types by increasing the ratio of washing soda to calcium hypochlorite (HTH) used in making up the stock solution. Tests on metal strips indicated that the corrosiveness fell off rapidly as the ratio was increased from 1:1 to 1:3, and more slowly from then on. Germicidal tests upon *Str. lactis* showed, with the technique employed, no slowing down until ratios of 1:7 to 1:9 were reached. Can rinsing tests were then made in which a mildly alkaline (ratio 4:3) and a strongly alkaline (Diversol) solution were compared with one of intermediate alkalinity (ratio 1:8). While differences were less marked than in the laboratory tests, it was found that the solution of intermediate alkalinity was much more effective than Diversol and approached that of the mildly alkaline product (table 1).

Further studies were conducted some months later to check on the effect of adding much larger amounts of alkali as advocated by Quam[2], who suggests that corrosion may thus be eliminated without impairing germicidal efficiency. It was found that sodium carbonate could be added up to 1 per cent without any marked decrease in germicidal speed, while at 0·25 per cent corrosion was negligible. A solution containing tri-sodium phosphate at 0·1 per cent was more corrosive and showed a greater reduction in germicidal speed, while a solution

[1] Report of the Dom. Agr. Bacteriologist for the years 1929 and 1930. Dom. of Can. Dept. of Agr. 1931.
[2] Quam, G. N. Food Industries 2, 121-122, 1930.

containing sodium hydroxide at 0·1 per cent was slowed up very decidedly. It would seem therefore that where corrosion is feared the addition of sodium carbonate to the rinsing or spraying solution to give a concentration of 0·25 to 0·5 per cent would be desirable.[1]

TABLE 1.—CAN RINSING TESTS WITH HYPOCHLORITE SOLUTIONS OF DIFFERENT DEGREES OF ALKALINITY

| — | HTH and washing soda | | Diversol | Control |
|---|---|---|---|---|
| | Solution X (4 : 3) (mild alk.) | Solution K (1 : 8) (intermediate) | | |
| Number of cans examined | 31 | 32 | 32 | 8 |
| Average count, 1 cc. rinse | 41 | 74 | 581 | 55,460 |
| Median count, 1 cc. rinse | 38 | 66 | 395 | 49,500 |
| Average percentage survival | 0·074 | 0·133 | 1·05 | |
| *Distribution of counts—* | | | | |
| Below 10 per cent | 16·1 | 12·5 | 0·0 | 0·0 |
| 11–50 | 61·3 | 34·4 | 0·0 | 0·0 |
| 51–200 | 22·6 | 50·0 | 15·6 | 0·0 |
| 201–1,000 | 0·0 | 3·1 | 68·8 | 0·0 |
| over 1,000 | 0·0 | 0·0 | 15·6 | 100·0 |

Methods for evaluating the germicidal potency of chlorine solutions are badly needed. Studies by the Division with F.D.A. technique as recommended by Myers and Johnson[2] indicated that the excessive amounts of organic matter employed gave results unduly favourable to chloramine-T solutions and tended to minimize differences between hypochlorites of high and low alkalinity, while results were variable due to the prevalence of "skips" and "misses." The use of a standardized suspension of the test organism, and subculturing into litmus milk was an improvement but results were still irregular. Subculturing to an agar slant (Burri method) gave more uniform results.

All these methods demand more time and equipment than is available in many milk and other food plant laboratories, and a more simple, convenient method for ranking in order of potency a series of hypochlorite products would be extremely useful. Since all experience to date has indicated that at 20° C. the pH of the solution exerts a profoundly modifying effect upon germicidal speed (and corrosiveness) attempts were made to determine differences in degree of alkalinity by colorimetric methods. A method was devised using powdered phenolphthalein which gave results in close agreement with the actual pH values as determined by the glass electrode.[3]

When comparisons were first made between the relative ranking of twenty hypochlorite products by the indicator method and by the Burri slant method, certain discrepancies were discovered. These were found to be due to the lesser buffer capacity of certain products, which showed a much greater change in pH upon dilution from 100 p.p.m. available chlorine (the concentration employed in the indicator test) to 2 p.p.m. (as employed in the Burri slant test). It would therefore seem advisable that any tests for ranking a series of products in order of potency be conducted at the concentration of chlorine at which the solutions are to be employed in practice, as otherwise erroneous impressions may be obtained as to the comparative germicidal potency of certain products.

To meet the need for a biological method of testing which would approximate more nearly the conditions under which a chlorine solution is employed in practice, and to avoid the disadvantages mentioned in connection with the

[1] For further details see Johns, C. K., 20th Ann. Rept. Internat. Dairy and Milk Inspectors, 197-209, 1931.

[2] Myers, R. P., and Johnson, A. H. Proc. Internat. Assoc. Milk Dealers, Lab. Sect. 25, 21-55, 1932.

[3] For details see Johns, C. K. Sci. Agr., 14, 585-607. 1934.

methods previously discussed, studies were made with a method where the organism was present upon a surface in a film of diluted milk, and then exposed to the action of the hypochlorite solution while gently agitated. This method, known as the glass slide method, proved to be quite promising, possessing other advantages over the previously tested methods in addition to those already mentioned[1].

Although most workers have reported that added alkali depresses germicidal speed of hypochlorite solutions, Fabian, Beavens, Bryan and Jensen[2] reported that such additions increase germicidal speed against *Bact. coli* at 50° C. both in the presence and absence of added ice cream mix. During the course of the studies on testing methods reported above, an investigation was made of the apparent discrepancy between their results and those obtained here and at other laboratories. Tests were carried out with both *Bact. coli* and *Staph. aureus* at 20° and at 50°, using the milk tube technique, to measure the potency of ordinary sodium hypochlorite of mild alkalinity as compared to the same solution containing additions of 0·5 per cent sodium carbonate, trisodium phosphate and sodium metasilicate (metso). It was found that at 50° C. the added alkali did accelerate the destruction of *Bact. coli* as compared with the control, but tests with the alkali salts added to distilled water proved that this was a result of the combined action of pH and temperature and not to any stimulating action of the alkali upon the hypochlorite. At 20° C. the reverse held true, the added alkali depressing the germicidal action of the hypochlorite. The added alkali definitely depressed the germicidal action against *Staph. aureus* at both temperatures, and this fact, together with its greater resistance to chlorine, make it a more suitable test organism than *Bact. coli* for studies of this nature[3].

## Milking Machine Sterilization

The studies[4] on methods of caring for milking machines, with particular reference to simplicity, have been continued. On many farms the lack of an abundant supply of hot water for suction washing of the milk tube system has presented difficulties. Some tests in 1928 where a cold water rinse only was followed by various sterilizing treatments gave reasonably low counts, but such a method could not be recommended on account of the more rapid decline in the strength of hypochlorite soak solutions as well as deterioration of the rubber parts.

In 1930 Parfitt[5] reported good results from the use of weak lye solution on well washed milker tubes. Such solutions avoided the troublesome "sandy" deposit of calcium phosphate, etc., appearing on rubber when chlorine compounds are employed with very hard water. Several series of tests were run with this method, starting in August 1930, the results of which appear in table 2. After encouraging results with the lye solution (Series I) comparisons were made between it and hypochlorite (Series II). In all these tests the machines were suction rinsed with cold and then hot water, and then the milk tube system placed on solution racks and filled with the sterilizing solution.

The data for Series I indicate a slight superiority of lye over hypochlorite. As lye possesses valuable detergent properties dissolving casein and saponifying fat, it was tested following a cold water suction rinse in Series II and III, in which it was compared with hypochlorite and chloramine-T respectively. Comparing the bacterial counts the advantage in favour of the lye is fairly

[1] For details, see Johns, C. K. Sci. Agr. 14, 585-607. 1934.
[2] Ind. Eng. Chem. 23, 1169-1173, 1931.
[3] For details see Johns, C. K. Ind. Eng. Chem., 26, 787-788. 1934.
[4] Report of the Dominion Agricultural Bacteriologist for 1927-1928, p. 3-14. Also Dom. of Canada Dept. of Agr. Bul. No. 127, 1929.
[5] Parfitt, E. H. Purdue Agr. Exp. Sta. Bul. 348, 1931.

definite, while the lye was greatly superior from the standpoint of physical cleanliness, keeping the tubes free from the semi-solid residue so prevalent when chlorine was used without the preliminary hot water rinse.

TABLE 2.—SUMMARY OF BACTERIAL COUNTS ON MILK DRAWN BY MACHINES TREATED WITH VARIOUS STERILIZING SOLUTIONS

| Series | Treatment | Total number of counts | Percentage distribution of counts per c.c. | | | | |
|---|---|---|---|---|---|---|---|
| | | | < 1,000 | 1,001-5,000 | 5,001-10,000 | 10,001-20,000 | > 20,000 |
| I | *Hot water suction washing—* | | | | | | |
| | *(a)* Tubes filled with lye solution 0·6 per cent..... | 51 | 13·2 | 75·5 | 5·7 | 1·9 | ..... |
| | *(b)* Tubes filled with hypochlorite 200 p.p.m..... | 53 | 20·8 | 62·2 | 7·6 | 7·5 | 1·9 |
| II | *Cold water suction rinse—* | | | | | | |
| | *(a)* Tubes filled with lye solution 0·6 per cent..... | 141 | 3·6 | 72·4 | 15·2 | 3·6 | 5·1 |
| | *(b)* Tubes filled with hypochlorite 200 p.p.m..... | 139 | ..... | 46·1 | 41·7 | 10·1 | 2·1 |
| III | *Cold water suction rinse—* | | | | | | |
| | *(a)* Tubes filled with lye solution 0·5 per cent..... | 62 | ..... | 3·2 | 40·3 | 45·2 | 11·3 |
| | *(b)* Tubes filled with chloramine-T 200 p.p.m..... | 63 | ..... | 9·5 | 23·8 | 47·6 | 19·1 |
| IV | *Cold water suction rinse—* | | | | | | |
| | *(a)* Tubes filled with lye solution 0·4 per cent..... | 45 | ..... | ..... | 31·1 | 31·1 | 37·7 |
| | *(b)* Tubes filled with Metso solution 0·5 per cent..... | 45 | ..... | ..... | 15·6 | 40·0 | 44·4 |
| V | *Cold water suction rinse—* | | | | | | |
| | *(a)* Tubes filled with lye solution 0·4 per cent..... | 39 | ..... | 30·8 | 33·3 | 17·9 | 18·0 |
| | *(b)* Tubes filled with washing soda solution 1 per cent..... | 34 | ..... | 3·0 | 5·9 | 14·7 | 76·4 |

Because of the corrosive action of lye upon aluminum (which is used in the milk tube system of at least one machine) a series of tests was made comparing lye with sodium metasilicate (Metso), the latter being non-corrosive for aluminum (Series IV). Metso appears to be almost equally as effective as lye solution in its antiseptic properties, while not at all inferior in maintaining the physical cleanliness of the tubes.

Washing soda being safer to handle than lye, and subject to less deterioration in strength when exposed to the atmosphere, a series of tests was conducted comparing washing soda solution (1 per cent) with lye solution (0·4 per cent). The data appearing under Series V indicate that the alkalinity of the washing soda solution (pH approx. 11·3) is not sufficiently high to inhibit bacterial growth in the tubes to the same extent as the lye (pH approx. 12·6). No differences in physical cleanliness could be observed.

In several of the series of the tests reported above, in addition to total counts, estimations were made of the numbers of organisms of the coli-aerogenes group. The results indicated a superiority on the part of the lye solution over the other treatments in the destruction of the lactose-fermenting organisms, according with the results of more extensive tests described in the next section.

Several hundred samples of milk drawn by machines treated with lye solution have been examined with regard to the possible effect of traces of lye solution upon the flavour of the milk, but in no case has any off-flavour been detected which could reasonably be attributed to the use of the lye. Thus there seems to be no urgent need for subsequent rinsing of the tubes, and if this is considered desirable, only water of known sanitary quality should be used.

## Relative Efficiency of Various Methods of Sterilization of Utensils in the Destruction of *Bact. coli.*

While there is general agreement that the presence of *Bact. coli* in milk may not represent manurial pollution (since feed, grain and more important, unsterilized utensils are also sources) it is recognized that coli-aerogenes organisms are an undesirable group in milk and dairy products due to their ability to cause off-flavour and other defects. In addition there is evidence that, apart from the total bacterial count, the keeping quality of milk is directly affected by the number of *Bact. coli* present. During the course of our studies with chemical disinfectants, miscellaneous observations suggested the probability that compared with steam or hot water certain chlorine compounds were more efficient in reducing the total count than in destroying coliform organisms. The action of various sterilizing agents upon *Bact. coli* was therefore studied more closely.

(*a*) *Milking Machine Experiments.*—The milk tube system of five De Laval units, uniformly contaminated with milk having a high content of *Bact. coli* were maintained under five different methods of sterilization for a period of three weeks. After contamination with the milk culture the tubes drained for one hour, when a cold water rinse was given. This was followed by the sterilizing treatment. The treatments employed were

(1) Immersion in water 170° F. for 20 minutes, after which the tubes were hung up to dry.
(2) Tubes kept filled between milkings with sodium hypochlorite, 200 p.p.m.
(3) Tubes rinsed with sodium hypochlorite, then hung up to drain.
(4) Tubes kept filled between milkings with chloramine-T, 200 p.p.m.
(5) Tubes kept filled between milkings with lye solution, 0·4 per cent.

Shortly before the afternoon milking the machines were assembled and 4 liters of sterile water were "milked" from an artificial udder through each unit into sterile milker buckets. Where chlorine was employed, thiosulphate was added to the rinse to check further action of the chlorine. Total bacterial counts of the recovered rinse were made by plating on purple lactose agar. Quantitative estimations of *Bact. coli* were made by three methods: plating on ferrocyanide citrate agar, plating on MacConkey's agar and by the dilution method using lactose bile broth.

TABLE 3.—MILKING MACHINE TESTS. EFFECT OF DIFFERENT METHODS OF STERILIZING ON TOTAL BACTERIA AND *Bact. coli*

| Treatment | Bacteria counts (log. aver.) | |
|---|---|---|
| | Total | Bact. coli |
| Hot water immersion 170°F. 20 minutes | 9,890 | 0·47 |
| Sod. hypochlorite 200 p.p.m. (rack) | 8,590 | 132·0 |
| Sod. hypochlorite 200 p.p.m. used as rinse | 6,650 | 237·0 |
| Chloramine-T 200 p.p.m. (rack) | 10,350 | 10·9 |
| Lye (0·4 per cent solution) | 8,520 | 0·68 |

A summary of the average total counts and *Bact. coli* counts from the daily analyses over a three-week period is given in table 3, figures for the coli count being from the ferrocyanide citrate agar, which medium gave considerably the highest numbers. As regards total count there was no marked difference in the effectiveness of the various treatments. On the other hand striking differences were noted in the ability of the treatments to destroy *Bact. coli*, with hot water and lye showing the greatest destruction, the latter being by far the most effective of the chemical treatments.

(*b*) CAN RINSING EXPERIMENTS.—A similar comparison was also made of the germicidal action of sterilizing compounds when used as rinses for 8-gallon milk cans. Cans were rinsed with contaminated milk, drained and left to stand for two hours. After being washed in the usual manner, duplicate cans received sterilizing rinses in which treatment with scalding water was compared with sodium hypochlorite (200 p.p.m.), chloramine-T (200 p.p.m.) and lye (0·4 per cent). Two series, each three weeks, were run. In the first the cans were tested immediately after treatment, while in the second series the cans were allowed to stand 19 hours before rinsing and analysis. Thus both germicidal and growth inhibiting effects were measured.

In both series the chlorine rinses were the most efficient in reducing total contamination, hot water and lye being less effective. Against *Bact. coli*, however, lye again showed the strongest action, confirming the results with the milking machines. There also was evidence of some residual effect of the chemical treatments in inhibiting growth subsequent to treatment, which fact may be of some practical importance.

A comparison of methods, five in all, for the estimation of *Bact. coli* showed the ferrocyanide citrate agar to give the highest average, slightly greater than eosin-methylene blue agar. The average counts on a percentage basis were ferrocyanide citrate agar 100, eosin-methylene blue gar 97·5, lactose bile broth 81·2, gentian violet bile 55·8 and MacConkeys agar 51·5. Ferrocyanide citrate agar also showed a high degree of reliability as judged by confirmatory tests, 98·6 per cent of 654 colonies picked from plates giving positive results.

### Steam vs. Hypochlorite for the Sterilization of Milk Cans and Bottles

Steam under pressure, generally conceded to be the ideal means of sterilizing dairy utensils, is employed at the Central Experimental Farm dairy. In view of the increasing use of chlorine solutions as sterilizing rinses, an opportunity was taken to compare the efficiency of this method with that of steam sterilization. Starting August 3, 1932, treatment with a hypochlorite solution[1] (100 p.p.m. available chlorine) was alternated with the steam sterilization (5 pounds pressure for 20 to 30 minutes). The efficiency of each method was judged in the following manner: Shortly before the afternoon milking six cans were each rinsed with 300 cc. of sterile water, and six pint bottles with 100 cc. water. (For the hypochlorite treatment, the rinse water contained sodium thiosulphate). The recovered rinse was then analysed.

The results of analysis of 141 cans and 143 bottles showed the hypochlorite to be somewhat less effective than steam under pressure, the difference being more marked with bottles than with cans. When, however, it is realized that the bacterial contamination of milk on filling the worst can would be 122 per cc. and from the worst bottle 165 per cc. the differences are seen to be relatively insignificant. Against this must be set the convenience and cheapness of the hypochlorite method, and the greater comfort of the man working in the wash-room, the temperature averaging over 7° F. higher when steam was used. It should also be noted that in all these tests the preparation of the solutions and the treatment of the utensils and bottles were carried out by non-technical help without any direct supervision, so that equally good results should be obtainable by any intelligent operator.

### Routine Dairy Control Work

In addition to the investigations reported above the Division maintained a bacteriological control of the dairy operations at the Central Experimental Farm. Each week two or three analyses were conducted on the raw milk, the

[1] Solutions of mild alkalinity, and the same solutions with 1 per cent washing soda added to reduce corrosiveness, were both used in these tests.

pasteurized milk in the vat and the same milk after bottling. In addition, surprise samples were taken from time to time to check on the methods employed by the milkers. Results were reported to the Division of Animal Husbandry, from which Division we have received constant co-operation in all our dairy bacteriological investigations.

## SOIL BACTERIOLOGY

### Legume Culture Distribution

The distribution of cultures for the inoculation of legume seed to Canadian farmers was continued during the years 1931-33. The distribution of "nitrocultures" is maintained for educational and experimental purposes, sufficient culture for the treatment of 60 pounds of legume seed being sent free to any farmer on the understanding that the user report the results. Our object is therefore (*a*) to encourage the more widespread practice of seed inoculation and (*b*) to accumulate data on the practical results of seed treatment under field conditions. During the years under review the numbers of cultures distributed were respectively 2,561, 6,143 and 7,557. The increases noted in 1932 and 1933 were largely due to our co-operation in the scheme of seed loans to farmers of the Prairie Provinces, whereby cultures sufficient to treat the legume seed distributed by Government aid were provided.

By means of reports returned by farmers using cultures, the Division has been accumulating information on the practical benefits accruing from inoculation in the opinion of the farmers themselves who are the ultimate judges of the value of any farm practice. Out of 1,799 reports so far received, 1,403 or 78 per cent indicated a benefit due to inoculation (81 per cent where crops were grown for the first time). In a large proportion of cases where no benefit was noted the treated and untreated crops grew equally well, indicating not a failure of the culture, but rather that the soil was already supplied with the proper strain of bacteria. On the other hand, the information from our reports supports the view that re-inoculation may be of value. The addition of a "good" strain of bacteria, of high nitrogen-fixing capacity or better suited to the soil type, rather than the mere presence of nodule-forming bacteria in the soil, is probably a factor of importance in such cases.

### Examination of Miscellaneous Inoculants

During the triennial period a number of legume inoculants appearing on the market were examined at the request of the Seed Branch, Department of Agriculture. The tests undertaken consisted in estimating the numbers of organisms appearing on Ashby's agar, while in addition greenhouse tests were made of the ability of the culture to produce nodules on the appropriate legume plants when applied as directed. Seeds were sown in sterile sand to which was added plant food minus nitrogen with controls of uninoculated seed and of seed inoculated with a pure culture of *Rhizobium* corresponding to that of the inoculant.

While most of the cultures were capable of inducing nodule formation on the appropriate legumes, the results indicated a superiority of the older method, whereby the seed is moistened when the culture is applied, over the dry inoculation method in which the culture, in the form of a finely divided powder, is simply mixed dry with the seed. Furthermore cultures containing bacteria for treating legumes of different cross-inoculating groups were unsatisfactory. Where two strains of *Rhizobium* are mixed in a single culture there is the probability of one strain being suppressed, and it is therefore recommended that an inoculant contain bacteria of but one cross-inoculation group.

In addition to the legume inoculants, the use of which is firmly based on sound principles and is a recognized aid to present-day farming, there have appeared from time to time on the market cultures purporting to aid the growth of all types of crops, non-legumes as well as legumes. A number of such preparations have been examined and previously reported upon in detail.[1]

In 1931 examination was made of a preparation called "Jermite" which was stated to be a "scientifically prepared bacterial compound in liquid form having remarkable plant invigorating properties" and said to be of value for both leguminous and non-leguminous plants, used as a seed inoculation. The retail price was $10 per gallon. The material was a thick, blackish opaque liquid with the appearance of sewage sludge. As might be expected the material contained large numbers of bacteria. As far as those types are concerned, however, which are of importance in soil in the transformation of nitrogenous compounds, specially nitrifying and nitrogen-fixing bacteria, the material was particularly deficient. Quantitative tests to estimate the nitrifying capacity of the product showed that this was less than that of an equivalent amount of soil.

Greenhouse tests, to note the effect of "Jermite" on plant growth were made using wheat, oats and one legume crop, peas. The results in all cases indicated no beneficial effect of the material over untreated plants as far as height of plants or weight of crop were concerned. In the case of the peas there was a distinct superiority of the plants treated with nitro-culture over those inoculated with "Jermite," which latter were similar to those without any treatment. From the examination made there was no evidence found that the material has any distinct worth, thus confirming the view that inoculation is of value only in the case of legumes treated with the proper cultures of nodule forming bacteria.

### Alfalfa Inoculation under Field Conditions. Effect of Inoculating Seed in Advance of Seeding

Studies of the viability of legume bacteria on inoculated seed, carried out several years ago by this Division under laboratory and greenhouse conditions, showed that alfalfa seed, inoculated with a culture of *Rhizobium meliloti*, retained numbers of viable organisms after six months' storage which were capable of producing nodules. Although the effect of inoculation was noticeable after 6 months, yet the nodule-forming capacity of the treated seed was found to decrease immediately on storage, most rapidly during the first few weeks. Even after 24 hours' storage, a decrease in nodule forming power was observed, accompanied by a pronounced decline in the numbers of living bacteria on the seed coats.

To test the effect under field conditions of inoculating seed at intervals in advance of seeding, experiments have been under way since 1928 at the Beaverlodge Experimental Station.[2] In ordinary practice it is frequently necessary to delay seeding after treatment and the tests therefore had a distinct practical bearing. Alfalfa plots were seeded in quadruplicate using seed which had been treated respectively immediately, 24 hours, one week and two weeks in advance, using the soil-glue method, with soil from an area which had previously supported good growth of alfalfa.

Results of the 1928 seeding indicated that holding inoculated seed before sowing was not detrimental, indeed the highest yields resulted from seed treated one week in advance of seeding (*see* table 4). This was evident, not only the first crop year, but also during the second and third crop years. Considering the total hay per acre obtained in three years, the plots sown with seed treated immediately before seeding gave an increase of but 1·5 per cent over the controls. Plots with seed inoculated 24 hours, one week and two weeks in advance gave

[1] Reports of Dominion Agricultural Bacteriologist for 1925 and for 1929-30.

[2] See also Reports of Supt., Dom. Exp. Sub-station, Beaverlodge, Alta., for 1929 and following years.

increased yields over controls of 14·1 per cent, 22·3 per cent and 14·9 per cent respectively. Since the orthodox recommendations have always stressed the necessity of sowing immediately after inoculation, the results of this field test were indeed surprising, and warranted repetition.

TABLE 4.—INOCULATION OF ALFALFA AT INTERVALS IN ADVANCE OF SEEDING (BEAVERLODGE)

| — | Hay per acre | | | | Increase over control |
|---|---|---|---|---|---|
| | lb. 1929 | lb. 1930 | lb. 1931 | lb. total | % |
| *1928 seeding—soil inoculation—* | | | | | |
| No inoculation | 2,435 | 3,545 | 2,847 | 8,827 | ..... |
| Inoculation immediately before sowing | 2,340 | 3,806 | 2,813 | 8,959 | 1·5 |
| Inoculation 24 hours before sowing | 3,027 | 3,927 | 3,119 | 10,073 | 14·1 |
| Inoculation 1 week before sowing | 3,207 | 4,406 | 3,185 | 10,798 | 22·3 |
| Inoculation 2 weeks before sowing | 2,926 | 4,077 | 3,141 | 10,164 | 14·9 |
| *1930 seeding—soil inoculation—* | | | | | |
| | 1931 | 1932 | 1933 | total | |
| No inoculation | 1,075 | 607 | 1,630 | 3,312 | ..... |
| Inoculation immediately before sowing | 1,105 | 1,499 | 2,787 | 5,391 | 62·7 |
| Inoculation 24 hours before sowing | 1,134 | 1,513 | 2,846 | 5,493 | 65·8 |
| Inoculation 1 week before sowing | 996 | 1,580 | 2,725 | 5,301 | 60·1 |
| Inoculation 2 weeks before sowing | 933 | 1,391 | 2,397 | 4,721 | 42·5 |
| *1930 seeding—culture inoculation—* | 1931 | 1932 | 1933 | total | |
| No inoculation | 1,075 | 607 | 1,630 | 3,312 | ..... |
| Inoculation immediately before sowing | 943 | 759 | 2,061 | 3,763 | 13·6 |
| Inoculation 24 hours before sowing | 952 | 946 | 2,245 | 4,143 | 25·1 |
| Inoculation 1 week before sowing | 809 | 827 | 2,061 | 3,697 | 11·7 |
| Inoculation 2 weeks before sowing | 779 | 860 | 2,201 | 3,840 | 15·9 |

The experiment was repeated in 1930, and amplified to include inoculation by two methods, pure culture inoculation in addition to the soil method. Results in general confirmed those of the previous trials. Yield data for the three crop years 1931-33 inclusive indicated that inoculation in all cases was of benefit, even where the seed was held as long as two weeks before sowing. (*See* table 4.) In the soil inoculation series the plots sown with seed inoculated immediately before sowing outyielded the uninoculated plots by 63 per cent. Plots sown with seed held 24 hours, one week and two weeks before seeding gave increases of 66 per cent, 60 per cent and 42 per cent respectively over the controls. In the series in which pure culture inoculation was used, the increases over the uninoculated plots were, for the same method of seed treatment, 14 per cent (immediate), 25 per cent (24 hours), 12 per cent (1 week) and 16 per cent (two weeks). Although inoculation in all cases was effective, the soil method proved superior to the pure culture method. The explanation lies in a better adaptation of the acclimated organisms of the soil inoculation than those of the pure culture to the particular soil conditions of the region. There was also evidence, from laboratory studies of isolated strains that the *Rhizobium* types used for the soil inoculation were physiologically efficient nitrogen-fixing organisms.

It was of interest to note that the advantage of inoculation in all cases was not apparent until the second crop year (delayed action). Considering this year alone, the soil inoculated plots as a whole showed an increase of 146 per cent over the controls, while with the culture inoculation series the increased yield was 40 per cent.

The results of the tests reported are of much practical significance in legume cultivation. Contrary to prevailing opinion, based largely on analogy from laboratory and greenhouse tests of nodule production, which holds that immediate sowing of treated seed is essential, the experiments show that holding inoculated seed for as long as a week or more does not necessarily lessen the effectiveness of the treatment.

### The Occurrence and Activity of Azotobacter and Rhizobium in Soils as Influenced by Season, Crops and Fertilizer Treatment

In November, 1931, an investigation was commenced with the object of studying the nitrogen-fixing bacteria in soils, particularly *Azotobacter* and *Rhizobium*. The special purpose was to note the quantitative and qualitative differences with respect to these important groups of organisms as influenced by the season, cropping and fertilizer treatment. Monthly samples of soil from areas which for 20 years have received respectively no fertilizer, farmyard manure, and artificial fertilizers are being taken for four consecutive years to cover one full rotation.

Results from two years' study have shown that the soil receiving no fertilizer contains consistently the highest numbers of *Azotobacter* colonies. There is, moreover, some evidence to show that *Azotobacter* from this area is in a higher state of physiological efficiency than in the soils from the areas receiving farmyard manure or artificial fertilizer. Apparently there is some compensation for lessened fertility due to nitrogen removal without fertilizer additions, through increased activity of the non-symbiotic nitrogen-fixing bacteria.

Quantitative studies of the symbiotic nitrogen-fixing bacteria associated with groups of legume crops have been made on *Rhizobium melilloti* (alfalfa, sweet clover, etc.), *R. leguminosarum* (vetch, pea, etc.), and *R. trifolii* (Red clover group). Although host plants of the first two types have not grown on the soil for at least twenty years, these have been consistently present in all soils studied. Contrary to the results with *Azotobacter* their numbers have been much lower in the unfertilized soil than in the soils receiving either manure or artificial fertilizers. As red clover is a normal crop in the rotation studied, the numbers of the corresponding nodule bacteria, *Rhizobium trifolii*, are naturally greater than those of the other types. No quantitative differences are noted, however, in the three soils studied.

## STUDIES OF HONEY FERMENTATION

In continuation of previous studies already reported by this Division[1], further investigations on honey fermentation were conducted in association with the Bee Division. In this work the National Research Council of Canada also co-operated through the appointment of Miss L. Farrell for research work in the field from June, 1929, to September, 1931.

### Types of Sugar-tolerant Yeasts Found in Normal Honey and Their Relation to Fermentation

In a previous study of the quantitative infection of normal honey by sugar-tolerant yeasts it was found that the tendency to ferment during subsequent storage increased with increasing yeast infection. To note whether the types of yeasts, as distinct from the numbers present, are of importance in causing fermentation, a study was made of 163 samples of normal Canadian honey representing all parts of Canada. The yeast types occurring most abundantly in the fresh samples were first determined. The honeys were then placed in storage in the Bee Division and examined to note fermentation. From those which fermented within a 14-month storage period the most abundant types were again isolated, studied and identified. It was thus possible to determine the types of yeasts most prevalent in fresh honey and those which are the active agents in causing spoilage during storage.

A comparison of 201 strains of sugar-tolerant yeasts obtained from the 163 normal samples showed that they represented eight different species, of which six were classified as *Zygosaccharomyces*, one as *Schizosaccharomyces* and one

[1] Reports of the Dominion Agricultural Bacteriologist for 1927-28 and for 1929-30.

as *Torula*. The frequency with which these types were found as the predominating organism varied widely. *Zygosaccharomyces richteri* was found to be the most ubiquitous, occuring as the predominant type in 120 samples. The second most numerous yeast, *Z. nussbaumeri*, occurred 23 times, the others less frequently, two types being isolated in but one instance each.

It was noted, after examining for signs of fermentation for 14 months, that in the case of only three yeast types, representing a small fraction of the samples, did the honeys in question all remain free from fermentation. Moreover in all cases of fermentation (37 samples) the originally predominant organisms were found to be *Zygosaccharomyces*. The belief that members of this genus are specially concerned with fermentation is supported by the results of the examination of the fermented honeys. In every instance the type of yeast predominating in the samples after spoilage proved to be of the genus *Zygosaccharomyces*.

It was observed that in many cases the yeast type predominating originally did not prove to be that most abundant after fermentation, leading to the belief that the organism with which a sample of honey may be most heavily infected is not necessarily the causal agent in fermentation. On the other hand *Z. richteri*, found as the predominant type in 120 samples, of which 22 later fermented, was found after fermentation not only in 20 of these cases but also in 9 samples in which the originally predominant yeasts were of other species. This yeast, first isolated from fermented honey, but also found as the most common yeast infecting hive nectar, has been likewise isolated from the nectar of various flowers and from apiary soil. Altogether it appears to be the most ubiquitous sugar-tolerant yeast concerned with honey.

The eight species of yeasts isolated from normal honey were studied in detail as to morphology, cultural characteristics and physiological properties, details of which may be found elsewhere[1]. It was a matter of considerable interest to find *Schizosaccharomyces octosporus* as one of the types in Canadian honey, since members of the genus are mainly tropical yeasts.

## A Bioactivator in Honey Stimulating Fermentation by Sugar-tolerant Yeasts

Studies on sugar-tolerant yeasts capable of causing honey fermentation indicated that little or no fermentative action occurred in synthetic media containing fermentable sugars, and nutrient salts with suitable nitrogen supply. The observation that active fermentation could be induced by the addition of small amounts of honey, however, suggested the possible presence in honey of some unidentified factor affecting fermentation which might have some bearing upon honey spoilage under practical conditions. Experiments were therefore undertaken to establish the nature of this factor. A summary of the results is given here, the detailed data being available elsewhere[2].

Using a basic synthetic nutrient solution which approximated the composition of honey, it was found that the addition of relatively small proportions of honey had very marked effects on the fermentation caused by yeasts, as measured by the evolution of carbon dioxide. This stimulation rises sharply with increasing amounts of honey up to 20 per cent above which the addition of honey exerts little stimulating effect, even up to 100 per cent honey.

More detailed studies on the nature of this stimulating factor indicated that it affected by adsorption by charcoal though not by fuller's earth and is dialyzable. It is insoluble in ether and acetone, though soluble in 85 per cent alcohol. It resists ordinary boiling, is not volatile, and withstands autoclaving in acid solution though proving less stable in alkaline solution. It is noticeably affected by exposure to moderate dry heat. It is not present in the ash constituents of honey and is therefore considered to be organic in nature.

[1] Lochhead, A. G., and Farrell, Leone. Can. J. Research, 5: 665-672. 1931.
[2] Lochhead, A. G., and Farrell, Leone. Can. J. Research, 5: 529-538, 539-543. 1931

With the apparent adsorption of the activating material by charcoal, attempts were made to recover it by elution with various solvents, the most successful being 95 per cent alcohol. It was found that the material recovered by treating the charcoal with alcohol showed but a slight activation when added to the basic test solution. The stimulation of yeast fermentation, however, was quite pronounced when, in addition, the solution contained a small amount of the filtrate from the charcoal treated honey, which by itself was relatively inert. Further quantitative tests confirmed this evident separation of the active principle into two fractions.

The recognition of this bioactivator naturally suggested a possible relationship with accessory growth factors found to be necessary for the normal growth and metabolism of certain yeasts, and generally considered under the term "Bios." The properties of the substance here reported corresponded very closely with those of the "Bios" of Wildiers, the fractionation of which has also been effected. Through the kindness of Prof. W. Lash Miller of the University of Toronto a series of tests was arranged to compare the effect of our bioactivator with that of Bios I and Bios II of the Toronto workers. Results indicated that for the Toronto yeast (*Saccharomyces cerevisiæ*) our two fractions are qualitatively interchangeable with Bios I and II. Corresponding tests with a honey-fermenting yeast (*Zygosaccharomyces mellis*) with both Bios fractions and our bioactivator showed a different effect which indicated that although our filtrate from the charcoal treatment of honey contained Bios I (inosite) yet with our sugar-tolerant yeast the active principle in the filtrate is not inosite. This unknown substance, is apparently not essential for the growth of the Toronto yeast, though it is present in crude Bios II.

## The Chief Factors Affecting Fermentation under Ordinary Storage Conditions

In the Report of the Division for 1929 and 1930, an investigation was described dealing with the effect of yeast infection of normal honey on fermentation during storage. This work dealt with tests on 191 samples from the 1929 crop and showed a tendency of honey to ferment with increasing yeast infection. Apart from their moisture content, which was of great importance, the chemical composition of honey showed no definite relationship to spoilage. With the object of securing more information as to the yeast infection of normal honey, and additional light on factors influencing fermentation, a further study was made of 128 samples from the 1931 crop in co-operation with the Bee Division and the Division of Chemistry. Determinations were made of the yeast infection, moisture, total nitrogen and titratable acidity, while the honeys were graded for colour by the "Pfund" scale. In addition, a method of determining the "bioactivator" value was worked out, to compare the honeys as to their content of the factor stimulating yeast action described in the previous section of this report.[1]

In confirmation of the results for 1929, yeasts were present in every one of the 128 samples analysed, the infection varying from 1 to 1,000,000 per gram. There was a rather higher degree of contamination in 1931, in which year 26 per cent of the samples had more than 10,000 yeasts per gram as compared with 7 per cent for 1929. Observations on the duplicate samples which were held in storage for a year confirmed the view that the higher the initial yeast count the greater is the tendency to ferment.

Respecting the effect of moisture, nitrogen, titratable acidity, bioactivator content, and colour of the honeys the data indicated that moisture is the only factor, apart from yeast infection, which is significantly related to spoilage. Although considerable variations were found in the values obtained for the

[1] For details see Lochhead, A. G. Zentralbl.f. Bakt. II Abt. 88, 296-302. 1933.

nitrogen, acidity, bioactivator and colour, yet it appears that under practical conditions of storage these factors do not influence fermentation to any significant degree.

Spoilage by fermentation, during storage at ordinary temperature, appears to be conditioned essentially by two factors determinable in the honey before storage, namely moisture and yeast count. This is indicated by the combined data for the 319 samples of honey which were studied in 1929 and 1931. From the data it is possible to designate a probable zone of safety within which a given honey sample may be expected to keep free from spoilage within 12 months. This is indicated in table 5.

TABLE 5.—MOISTURE, YEAST INFECTION AND PROBABLE ZONE OF SAFETY FROM FERMENTATION WITHIN 12 MONTHS

| Moisture | Effect of yeast count |
|---|---|
| % | |
| below 17·1 | Safe irrespective of yeast count |
| 17·1–18·0 | Safe if yeast count does not exceed 1,000 |
| 18·1–19·0 | Safe if yeast count does not exceed 10 |
| 19·1–20·0 | Safe if yeast count does not exceed 1 |
| above 20 | Always danger of fermentation |

For the prevention of fermentation, storage at low temperature and heating have been most frequently advised. The heating of honey has certain objectionable features, particularly with respect to its effect on flavour and enzyme content, while low temperature cannot always be maintained up to the time the product is ultimately consumed. A knowledge of the fermenting tendency, then, as reflected by the moisture and yeast content, should be of value in determining which honeys may be regarded as free from danger of fermentation within one year, and which should be destined for quicker consumption or subjected to further treatment to prevent spoilage.

### The Effect of Preservatives in Preventing Honey Fermentation

In a previous report (1929-1930) work was described concerning the preservative effect of various substances in a honey solution inoculated with honey fermenting yeasts. Of the various compounds tested which prevented fermentation in concentrations within the limits prescribed by the Food and Drugs Act, sodium benzoate, sodium sulphite and sodium bisulphite were the most promising and appeared to merit further testing on a practical scale. Consequently the tests to be described were carried out with freshly extracted honey with the co-operation of the Bee Division.

EXPERIMENT I (1930 CROP).—The honey used had a moisture content of 19·0 per cent and a yeast count of 250,000 per gram, and was considered liable to rapid spoilage. Three lots were treated separately with 0·05 per cent sodium benzoate, 0·01 sodium sulphite and 0·025 per cent sodium bisulphite respectively while a fourth lot of 100 pounds was left untreated. The honey was then packed in 5-pound pails, 24 pails for each lot, and stored in an ordinary room subject to fluctuating temperatures. Periodic testing was made for appearance, fermentation and yeast count (see table 6).

The untreated honey had all fermented within 6 months' storage. The treated lots remained free from fermentation for two years with sharply decreasing yeast counts which indicated a destructive action of the preservatives used. The sulphite and bisulphite lots, however, showed an undesirable darkening in colour and were likewise inferior in flavour to the benzoate lot in which latter no adverse effect of the preservative could be noted. The superiority of sodium benzoate, which was used in but half the concentration permissible in foodstuffs, led to further work with the use of this compound in 1931.

TABLE 6.—EFFECT OF PRESERVATIVES ON HONEY

| 1930 experiment | Sodium benzoate 0·05 per cent | Sodium sulphite 0·01 per cent | Sodium bisulphite 0·01 per cent | Control |
|---|---|---|---|---|
| Yeast count per gram at start | 250,000 | 250,000 | 250,000 | 250,000 |
| 6 months | 180 | 1 | 0 | 400,000 |
| 9 months | 3 | 0 | 0 | 380,000 |
| Fermentation | −(2 yrs.) | −(2 yrs.) | −(2 yrs.) | +(6 months) |
| Flavour | good | off | off | fermented |
| Colour | good | dark | dark | ..... |

| 1931 experiment | Sodium benzoate | | | Control |
|---|---|---|---|---|
| | 0·05 per cent | 0·025 per cent | 0·01 per cent | |
| Yeast count per gram at start | 6,000 | 6,000 | 6,000 | 6,000 |
| 6 months | 25 | 130 | 990 | 44,500 |
| 9 months | 3 | 54 | 700 | 186,000 |
| 12 months | 2 | 9 | 4,470 | 79,400 |
| 15 months | 0·1 | 2 | 25,900 | 48,200 |
| 21 months | 0·03 | 0·06 | 10,500 | 18,600 |
| 30 months | 0·03 | 0·03 | 1,750 | 1,750 |
| Fermentation | −(2½ yrs.) | −(2½ yrs.) | +(18-21 mos.) | +(9 mos.) |
| Flavour | good | good | fermented | fermented |
| Colour | good | good | good | ..... |

EXPERIMENT II (1931).—Four lots of honey were prepared as before, three being treated with 0·05 per cent, 0·025 per cent and 0·01 per cent sodium benzoate respectively with an additional lot as control, 24 tins of honey of each lot being placed in storage as before. The honey showed a moisture content of 19·2 per cent with an original yeast count of 6,000 per gram.

The untreated tins all showed signs of fermentation after nine months' storage. Sodium benzoate in concentration of 0·05 and 0·025 per cent, exerted a distinctly depressing effect on the yeast numbers, which continued to show for 15 months, at which time these lots were practically sterile and naturally remained free from fermentation for more than two years. The honey treated with 0·01 per cent benzoate show a much slower decline in yeast numbers for 9 months, after which a rise in numbers occurred so that after 15 months traces of fermentation were manifest (see table 6).

The samples with 0·05 and 0·025 per cent benzoate were quite unaffected as regards flavour, colour and general appearance, though after 12 months the honey began to separate normally. The tests so far conducted indicate that sodium benzoate, in one-quarter the strength permissible in foodstuffs, will keep honey from fermenting without any abnormal effect on flavour or other characteristic.

## MISCELLANEOUS

### Co-operation in Fruit Products Investigations

This Division has been co-operating with the Horticultural Division in their cider investigational work, with particular reference to a study of methods for the production of a sterile, bottled product. For the elimination of micro-organisms capable of spoiling the appearance, flavour, etc. of bottled cider, particular attention has been given to the filtration process (Seitz filter) and to practicable methods of sterilization of bottles and crown seals.

Results to date indicate that with proper handling the Seitz EK filter is able to deliver consistently a sterile cider under the closed cuvée (pressure) system. Contamination from the air filter arrangement can, with adequate

facilities for steam sterilization, be likewise eliminated. The most serious source of contamination of the bottled product so far has come from the bottles themselves and the crown seals, consequently chief attention has been centred on these factors.

For the treatment of bottles, immersion in 3 per cent solution of flake caustic at 145-148° F. for 5 minutes was found to destroy yeasts, although leaving numerous moulds and bacterial spores still viable. Subsequent treatment with sodium hypochlorite (500 p.p.m. available chlorine) gave satisfactory results from the standpoint of sterility. Recent storage tests on the bottled cider, however, indicate that a sedimentation, due to physico-chemical effects is correlated with the strength of hypochlorite employed, and further work is underway with a view to the elimination of this storage defect through a determination of the strength of chlorine whch may be used with safety.

Co-operative studies on the general microbiology of cider fermentation have also been conducted. Chief attention so far has been paid to a comparison of yeast strains most suitable for cider making, from the standpoint of flavour and physiological efficiency as indicated by fermentative capacity, ability to ferment sugar solutions of high density etc. Studies are also underway to note the factors responsible for the darkening of cider which is occasionally noted particularly when apple juice concentrate is employed.

### Red Discoloration of Salted Hides

In September, 1932, the attention of the Division was called by Dr. W. E. Graham, of the National Research Council, engaged in leather research, to the question of the reddish discoloration frequently appearing on salted hides. This phenomenon, commonly referred to in the leather trade as "red heat," becomes evident on the flesh side of the hide during storage, and represents a defect which may occasion considerable loss to the industry through spotting and weakening of the fibre.

The appearance of the discoloration suggested a possible analogy to somewhat similar defects occurring on other salted animal products, notably codfish, caused by salt tolerant bacteria, types of which may be expected to be active on salted skins. Samples of hides were placed at our disposal by Dr. Graham, and work was carried out on the isolation and study of the causal and associated organisms. A detailed account is presented elsewhere.[1]

From Argentine hide a red halophilic sarcina was isolated as the causal agent. This organism, classified as *Sarcina litoralis* Poulsen, is similar to types found previously in association with red discoloration of fish, etc. Showing no proteolytic action it is regarded as capable of causing but little damage to hides.

From Canadian hides showing red discoloration, two species of pleomorphic rod-shaped organisms were isolated as active agents. One of these, occurring on salted cowhides, was found to be similar to *Serratia salinaria* (Harrison and Kennedy) Bergey et al., a source of reddening of cured codfish in eastern Canada. The other organism causing discoloration, isolated from buffalo hide, was regarded as a new species and designated *Serratia cutirubra* n.sp. Both of these organisms grow only in a medium containing at least 20 per cent salt, and are able to thrive in a saturated solution. Owing to their proteolytic action, they are considered capable of more damage to hide through injury to the fibre than the sarcinal types, which may also be present on Canadian hides. The source of the red organisms is believed to be the curing salts used.

In addition to the bacteria causing reddening, various types of non-chromogenic organisms were found, some of which showed proteolytic properties. The non-chromogenic types studied, however, though halophilic (salt-

[1] Lochhead, A. G. Can. J. Research, 10: 275-286. 1934.

loving), developed at a lower salt concentration range than the red organisms, showing no activity in a medium approaching saturation. They are therefore considered to be less active in causing injury to fibre in well-salted hides.

### General Analytical Service

In continuance of the policy maintained in former years, assistance was given, particularly to the farming community, through the examination of samples submitted for bacteriological analysis. During the three-year period under review, 1,448 samples were received for analysis, those submitted consisting of water from farm wells, milk and other dairy products, bread, honey, canned goods and other foodstuffs, legume inoculants, foulbrood specimens and others of an agricultural nature. Although planned to be subordinate to our investigatory work, this analytical service is often time-consuming, due to the special attention which the analysis of many samples requires. It appears, however, to be filling in increasing measure a needed service for our country population.

Co-operation was also given to the Fruit Branch, Department of Agriculture, through microscopic analyses of canned tomato products in connection with a survey conducted by that branch of Canadian canneries. Determinations of the mold count by the Howard method were made to indicate the quality of the raw product used for canning.

During the years 1931 to 1933, 494 samples of well waters were received for analysis and reported on as to their sanitary quality. Our findings indicated that 33·4 per cent were free of pollution, and 31·2 per cent definitely polluted, while 35·4 per cent were classed as suspicious. In general the results of the analyses have corresponded to those of former years, lending weight to the belief that with a considerable proportion of our farm wells the location and construction still leave much to be desired.

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