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A STUDY OF THE MICROFLORA OF ROOT BEER

A Thesis

Presented to

the Faculty of the Department of Biological Sciences

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

Leah Morford Senff

June 1963

This thesis is approved for recommendation to the Graduate Council.

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Ernest P. Edwards

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Dated July 23, 1962

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INTRODUCTION

Bacteria, yeasts, and molds associated with various beverages have been of interest for some time. Probably most intensively and extensively studied are those micro-organisms important in the production or spoilage of alcoholic beverages. The emphasis of these previous studies has been one directed toward the discovery and development of strains which will give good quality products in large amounts. Efforts also have been made to eliminate organisms responsible for undesirable effects (souring, cloudiness, etc.,). These efforts have resulted in the development of controlled techniques of inoculation and careful procedure throughout market preparation.

Carbonated beverages, however, are not products of microbial activity, and thus, microbiological study of such beverages has been concerned for the most part with other economically important aspects. Organisms considered are those which decrease the product's market value by harming the taste or appearance and those which might be pathogenic to the consumer.

Literature Review

Cloudiness, ropiness, and off-flavors are types of spoilage associated with soft drinks. Cloudiness

may result from the growth of bacteria or yeasts, the latter being more often the cause of such spoilage. One worker (McKelvey, 1926) found yeasts implicated in 85 per cent of 1500 spoiled samples of carbonated beverages. The condition of ropiness is most often associated with the presence of encapsulated members of the genus Bacillus (Tanner, 1944). Bacteria also may produce off-flavors in soft drinks; Lehman and Byrd (1953) reported a member of the genus Achromobacter which was responsible for musty odor and flavor in root beer.

Some work has been done with pathogenic organisms and their survival in carbonated beverages. Young and Sherwood (1911) investigated the length of time various pathogenic forms remained viable in soft drinks. They noted marked reduction in the number of inoculated organisms after 244 hours in an uncarbonated lemon syrup. The bacteria were not completely killed, however, throughout the time of the experiment. Koser and Skinner (1922) found that pathogenic forms such as Salmonella schottmulleri were killed more quickly than Escherichia coli. Zynovskii (1937) made inoculations with Salmonella typhosa and other pathogenic forms; he found that the bacteria survived only a few days in the beverage.

Various investigators have studied the suitability of the environment provided by soft drinks for microbial growth: Donald, et al. (1924) studied the effects of CO_2 on bacteria. Working with carbonated and uncarbonated ginger ale, they found that only the latter allowed bacterial growth. Other workers have employed artificial systems simulating carbonated beverages: Shillinglaw and Levine (1943) studied the effects of acidic conditions on various species of bacteria; Insalata (1952) studied the bactericidal effects of CO_2. Eagon and Green (1957) directed their efforts similarly, using various commercial soft drinks in their work. They noted that forms such as E. coli and Micrococcus pyogenes var. aureus were killed within a few days after inoculation.

Other workers have been concerned mainly with the number of bacteria present in soft drinks. Young and Sherwood (1911) noted that the number of organisms normally in the beverages was "extremely small".

Stokes (1920) found a varying number of bacteria in beverages he investigated. He concluded that dirty bottles were responsible for these results. Kilcourse (1923) found from 2 to 500 organisms/ml of soft drink, the average being 100 bacteria/ml. Formon (1925) found "few" bacteria in 60 samples of soda water.

Little effort has been made to determine the identity of organisms normally found in carbonated beverages. Gauscher and Geors (1915) found members of the genus Bacillus.

Statement of the Problem

The purpose of the present work was three-fold: (1) to determine the number of micro-organisms found in root beer ready for consumer use, (2) to study the effects of various temperatures and durations of incubation on this microbial population, and (3) to characterize the predominate species of contaminating bacteria.

MATERIALS AND METHODS

Bottles of root beer were obtained from Belfast Beverages, Inc., in San Francisco. All media was prepared during the course of the work from Difco dehydrated bases.

The work was divided into three experiments, each designed to determine the effects of different temperatures and durations of incubation. The same procedure was followed each time. At the beginning of the experiment a shipment of root beer was received, and the bottles were divided into three groups of 12 to 16 bottles. One group was incubated at an average temperature of 4° C (range 2.0 to 5.0° C), one at an average of 24° C (range 22.0 to 26.5° C), and one at an average of 34° C (range 31.0 to 37.0° C).

Each day, during the course of the experiment, three bottles were selected, one from each incubation temperature. The outside of each bottle was sponged with two per cent phenol and then was lightly flamed. After removal of the cap, the mouth of the bottle was flamed and a sterile 10 ml pipette was inserted. The pipette was allowed to remain in the bottle five to eight minutes while the CO_2 was eliminated. Then a one ml sample of root beer was pipetted off and into

a test tube containing 10 ml of melted, cooled agar (45° C). Each tube of inoculated agar was poured into a petri dish which was incubated at 31° C. Controls were poured also. The pH of the root beer was taken at this time also.

Daily checks of each plate were made for four days. Colonies were counted, and during the third experiment some representative colonies were chosen for further work. These colonies were repeatedly isolated through the pour plate and streak plate methods. When it was certain that pure cultures were obtained, the characteristics of the organisms were studied.

Several stains were used, including the Gram stain, the Capsule stain (Maneval's stain), the Spore stain (Dorner's method), and the negative stain (Dorner's nigrosin). All of the staining procedures were taken from Laboratory Manual for General Bacteriology (Peltier et al., 1959).

Measurements of the organisms were made from gram stains. Motility checks were made by observing hanging drop preparations; flagellar stains were not attempted.

Tests to determine biochemical abilities of the organisms were also performed. Media used in this part

of the work included the following:

1. Carbohydrates
   glucose
   lactose
   sucrose
   maltose
   mannitol
   sorbitol

2. Litmus Milk

3. Lead Acetate Agar

4. Nitrate Broth

5. Nutrient Broth

6. Nutrient Agar.

RESULTS

Similar results were obtained from each of the three experiments on the number and kinds of organisms found in the root beer. Initially, there were very few forms present, and as the work proceeded, little change in the number of organisms was observed. In the final experiment, which ran approximately two weeks, only eight bacterial colonies and one mold were isolated (Table I).

It was difficult, with such a small number of organisms, to ascertain the effects of the different temperatures and times of incubation. Table I shows the following general tendencies. The greatest number of organisms was isolated from the bottles stored at the coolest temperature. All organisms isolated from the soft drink at 37° C appeared within a few days of the beginning of the experiment; the 24° C samples yielded organisms until about one-half way through the work; and bacteria appeared in the 3° C samples throughout the period of experimentation.

The organisms isolated were all gram positive or gram variable, and the rod shaped forms predominated. Those bacteria chosen for further work are discussed later.

TABLE I

NUMBER OF ORGANIMS ISOLATED DURING
THIRD SET OF EXPERIMENTS

Day	Incubation Temperature		
	34°C	24°C	4°C
1	0	1*	1
2	2	0	0
3	0	0	0
4	1 mold	0	1
5	0	0	0
6	0	0	0
7	0	1	0
8	0	0	1
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	1

(*) numbers refer to bacterial colonies unless otherwise indicated

The pH of the root beer did not change throughout the period of experimentation, being 4.5 to 5.0. It was noted that bottles incubated at the higher temperatures evidenced greater gas pressure than those at lower temperatures.

The characterization of five "species" of bacteria was carried out, and the results of this work are summarized, (Tables II through VI). The bacteria are designated by number: "4", "5", "6", "8", and "16".

Morphologically, all of these bacteria except "6" were large rods; "6" was rounded, its form varying from spherical to oval. Gram reactions of all these micro-organisms were positive, although "5" did show some gram variability.

Two of the rods, "5" and "16", were spore-formers. Organism "5" had terminal spores which bulged the end of the cell giving it a matchstick appearance, while organism "16", a very large rod, exhibited sub-terminal spores which did not noticeably change the outline of the cell. The rods all exhibited definite capsules; the rounded forms had only a slight clear area about the cells.

The organisms varied greatly in the various growth characteristics of colonies, of streaks and of broth (Tables II - VI).

Biochemically, all organisms were relatively active, (Tables II - VI). All organisms, except "5", were capable of reducing nitrates to nitrites. None produced H_2S. In litmus milk, all bacteria gave a basic reaction and showed ability to bring about reduction; not all forms were capable of peptonization.

All five organisms were mesophils, growing well at temperatures between 25° C and 34° C. All but the rounded form were facultative with regard to O_2 requirements; organism "6" was an aerobe.

TABLE II

CHARACTERIZATION OF ORGANISM "4"

GROWTH CHARACTERISTICS

Nutrient Broth
- amount: moderate
- surface: grey pellicle
- sub-surface: -
- sediment: -

Agar Colonies
- form: irregular
- elevation: raised
- surface: smooth
- margin: undulate-lobate
- density: translucent

Agar Slant
- amount: abundant
- form: echinulate
- consistency: viscid

BIOCHEMICAL CHARACTERISTICS

Carbohydrates

Day	gluc	lac	suc	mal	mann	sorb
1	A	NA	✗	NA	✗	NA
2	A	NA	✗	NA	✗	NA
3	A	NA	A	NA	✗	NA
4						A

Litmus Milk

Day	pH	curd	peptonization	reduction
1	7	-	+ at top	-
2	7	-	+ at top	+ at bottom
3	7	-	+	+ at bottom
4				

Nitrate Reduction and **H_2S**

Day	to NO_2^-	NH_3	H_2S
1	+	-	-
2	+	-	-
3	+	-	-
4	+	-	-

O_2 and Temperature Relation

facultative mesophil

STAINING CHARACTERISTICS

Gram Stain

slant: G+ rods, no chains or spores. 5.29 x 0.75 μ.

broth: G+ rods, most cells in chains, no spores.

Spore Stain

negative

Capsule Stain

capsules present

Miscellaneous

single cells and pairs motile, longer chains non motile.

Key: + = positive result; - = negative result; G+ = gram positive; G- = gram negative; A = acid produced; ✗ = partial acid; NA = no acid

TABLE III

CHARACTERIZATION OF ORGANISM "5"

GROWTH CHARACTERISTICS

Nutrient Broth

amount:	moderate
surface:	"cottony"
sub-surface:	-
sediment:	"cottony"

Agar Colonies

form:	circular-irregular
elevation:	flat
surface:	smooth
margin:	lobate-erose
density:	translucent

Agar Slant

amount:	moderate
form:	effuse
consistency:	butyrous

BIOCHEMICAL CHARACTERISTICS

Carbohydrates

Day	gluc	lac	suc	mal	mann	sorb
1	A	NA	NA	A	NA	NA
2	A	✗	A	A	NA	NA
3	A	A	A	A	NA	NA
4					NA	NA

Litmus Milk

Day	pH	curd	peptonization	reduction
1	7	-	-	+
2	7	-	-	+
3	7	-	-	+
4				

Nitrate Reduction | **H_2S**

Day	to NO_2^-	NH_3	
1	-	-	-
2	-	-	-
3			-
4			

O_2 and Temperature Relation

facultative mesophil

STAINING CHARACTERISTICS

Gram Stain

slant: mostly G+ rods a few G-, some cells with spores, no chains 5.18 x 0.75μ .
broth: G+ rods, spores, some chains, some cells lie side by side.

Spore Stain

terminal, ellipsoidal spores, cells have "match-stick" shape

Capsule Stain

capsule present

Miscellaneous

non motile

Key: + = positive result; - = negative result; G+ = gram positive; G- = gram negative; A = acid produced; ✗ = partial acid; NA = no acid

TABLE IV

CHARACTERIZATION OF ORGANISM "6"

GROWTH CHARACTERISTICS

Nutrient Broth

amount:	moderate
surface:	-
sub-surface:	turbid
sediment:	granular

Agar Colonies

form:	circular, small
elevation:	raised, convex
surface:	smooth
margin:	entire
density:	opaque

Agar Slant

amount:	moderate
form:	beaded
consistency:	butyrous

BIOCHEMICAL CHARACTERISTICS

Carbohydrates

Day	gluc	lac	suc	mal	mann	sorb
1	✗	A	NA	A	NA	NA
2	A	A	NA	A	NA	NA
3	A	A	NA	A	NA	NA
4					NA	NA

Litmus Milk

Day	pH	curd	peptonization	reduction
1	7	-	-	-
2	7	-	-	+ at bottom
3	7	-	-	+ at bottom
4	7	-	-	+ at bottom

Nitrate Reduction

Day	to NO_2^-	NH_3
1	+	-
2	+	-
3	+	-
4		

H_2S

-
-
-
-

O_2 and Temperature Relation

aerobic mesophil

STAINING CHARACTERISTICS

Gram Stain

slant: G+ round forms, mostly paired, some in clumps. ave. diam. 1.65 μ

broth: G+ round forms, size variable.

Spore Stain

negative

Capsule Stain

thin clear area around cells.

Miscellaneous

morphology of cells varies from spherical to oval forms.

Key: + = positive result; - = negative result; G+ = gram positive; G- = gram negative; A = acid produced; ✗ = partial acid; NA = no acid

TABLE V

CHARACTERIZATION OF ORGANISM "8"

GROWTH CHARACTERISTICS

Nutrient Broth

amount:	moderate
surface:	grey pellicle
sub-surface:	-
sediment:	-

Agar Colonies

form:	irregular
elevation:	flat
surface:	smooth
margin:	undulate
density:	opaque

Agar Slant

amount:	abundant
form:	echinulate
consistency:	viscid

BIOCHEMICAL CHARACTERISTICS

Carbohydrates

Day	gluc	lac	suc	mal	mann	sorb
1	A	NA	✗	NA	NA	NA
2	A	NA	A	NA	✗	NA
3	A	NA	A	✗	✗	NA
4				A	A	NA

Litmus Milk

Day	pH	curd	peptonization	reduction
1	7	-	+ at top	-
2	7	-	+ at top	+ at bottom
3	7	-	+ at top	+ at bottom
4	7	-		+ at bottom

Nitrate Reduction		H_2S
Day	to NO_2^-	NH_3	
1	+	-	-
2	+	-	-
3	+	-	-
4	+		

O_2 and Temperature Relation

facultative mesophil

STAINING CHARACTERISTICS

Gram Stain

slant: G+ rods, no chains or spores, variable size. 3.79 x 0.75 μ.

broth: G+ rods, single cells or short chains (2-4 cells), no spores.

Spore Stain

negative

Capsule Stain

positive

Miscellaneous

another type of colony observed, rough surface, curled margin.

Key: + = positive result; - = negative result; G+ = gram positive; G- = gram negative; A = acid produced; ✗ = partial acid; NA = no acid

TABLE VI

CHARACTERIZATION OF ORGANISM "16"

GROWTH CHARACTERISTICS

Nutrient Broth

amount:	moderate
surface:	ring
sub-surface:	turbid
sediment:	-

Agar Colonies

form:	irregular
elevation:	flat
surface:	textured
margin:	undulate, curled
density:	opaque

Agar Slant

amount:	abundant
form:	echinulate
consistency:	butyrous

BIOCHEMICAL CHARACTERISTICS

Carbohydrates

Day	gluc	lac	suc	mal	mann	sorb
1	A	A	A	A	NA	NA
2	A	A	A	A	NA	NA
3	A	A	A	A	NA	NA
4					✗	NA

Litmus Milk

Day	pH	curd	peptonization	reduction
1	7	-	+ at top	-
2	7	-	+ at top	+
3	7	-	+ at top	+
4				

Nitrate Reduction and H_2S

Day	to NO_2^-	NH_3	H_2S
1	+	-	-
2	+	-	-
3	+	-	-
4	+	-	+

O_2 and Temperature Relation

facultative mesophil

STAINING CHARACTERISTICS

Gram Stain

slant: G+ rods, cells show internal clear area (spores) 5.7 x 1.5μ.

broth: G+ rods, mostly single cells, a few in pairs.

Spore Stain

sub-terminal, ellipsoidal spores.

Capsule Stain

capsule present

Miscellaneous

some colonies show swirls of growth off edges (due to motility)

Key: + = positive result; - = negative result; G+ = gram positive; G- = gram negative; A = acid produced; ✗ = partial acid; NA = no acid

DISCUSSION

Three aspects of this work will be considered in the discussion: (1) the sources of microbial contamination of the soft drink, (2) the conditions contributing to the small number of organisms found in the root beer, and (3) the tentative classification of organisms "4", "5", "6", "8", and "16".

Sources of Microbial Contamination

Root beer is a carbonated beverage, the main ingredients of which are root beer flavor, water, and syrup. The latter consists of sugar (sucrose), invert sugar, and dextrose (Jacobs, 1958). "Edible" organic acids and caramel color may or may not be included. The flavor (or concentrate) consists generally of oil of sassafrass and methyl salicylate or of oil of wintergreen or of oil of sweet birch or of a combination of these oils (Jacobs, 1958). Each of the above mentioned ingredients may contribute to the microbial content of the final product (Frazier, 1958). Preparation of the soft drink, which includes such operations as water filtration, syrup addition, carbonation, and capping, provides multiple opportunities for the invasion of micro-organisms (Insalata, 1956).

The water used in the soft drink manufacture is especially important. Drinking water, although sanitary, may be unsuitable; e.g., micro-organisms not harmful to man may gain access to the beverage and bring about conditions such as ropiness. Therefore, it is recommended that the water used be completely sterile (Sliger, 1956). Filtration is one means of bringing about sterility; neglected filters, however, may actually serve to introduce more organisms (Frazier, 1958).

Sugar may be another source of contaminants. Commercial processing of sugar (heating, clarification, evaporation, and crystallization) greatly reduces the number of organisms present. Further treatment of the sugar may be required, however, before use in soft drink preparation. The Bottlers of Carbonated Beverages have recommended tentative standards for sugar: $\leq$ 100 mesophils/10 grams and $\leq$ 10 yeasts or molds/10 grams (Frazier, 1958).

The equipment, bottles and closures all provide innumerable sites for the presence of micro-organisms; workers themselves may also be potential sources of contamination. Even the air in the building may carry micro-organisms into the product: Insalata (1956) mentioned yeast contamination which occurred via air currents circulating about returned, unwashed bottles stored in the plant.

Conditions Contributing to the Small Number of Organisms

The results of this experimentation indicate that, even with all these opportunities for contamination, the number of micro-organisms occurring in the root beer is very low. These results correspond with those of other workers, who generally have found that the bacterial content of carbonated beverages decreases with age of the drink until relative sterility is attained (Insalata, 1952). There are two main reasons for the small microbial population: (1) the environment provided by the soft drink, and (2) the sanitary restrictions observed in the manufacture of the product.

The fitness of a particular environment depends upon factors such as the availability of nutrients and water, the hydrogen ion concentration, and the temperature.

Root beer contains a fairly large amount of sugar, about 9.9 per cent Brix (Jacobs, 1951), and it is approximately 95 per cent water (Sliger, 1956). Therefore, it provides adequate energy-yielding sources and moisture. The osmotic pressure, however, may well be inhibitory to some micro-organisms.

The addition of "edible" acids and the practice of carbonation serve to give a high hydrogen ion concentration (Frazier, 1958), and many investigators

have been interested in the germicidal properties of this combination. Shillinglaw and Levine (1943) worked with 0.02 N solutions of acids often included in soft drinks (tartaric, phosphoric, and citric); phosphoric acid is often included in beverages like root beer (Jacobs, 1951). Shillinglaw and Levine (1943) found that intestinal organisms were killed within a few hours, and the addition of CO_2 or sucrose to the acid solutions increased the death rate.

Work directed toward determining the effects of CO_2 alone has revealed that the gas exerts a selective action on bacteria; "some are killed, others are not harmed" (Tanner, 1944). Insalata (1952) found that, in general, the higher the pressure of CO_2 used, the faster sterility occurred in a sugar solution. He also noted that some organisms could sustain themselves despite the presence of CO_2. He concluded that pH, Brix, and incubation time were key factors in determining whether or not organisms would remain viable in carbonated beverages.

Hagon and Green (1957) performed experiments to determine the effect of carbonated beverages per se on bacteria. Using beverages with pH 2.5 to 3.0 and about 3.5 volumes of CO_2, they found that organisms

such as Micrococcus pyogenes var. aureus survived not longer than 24 hours. This was true regardless of the incubation temperature: 4°, 25°, or 37° C. These same workers obtained similar results with non-carbonated beverage. Carbonation, therefore, seemed to increase the detrimental effects of the soft drinks but slightly.

Experimental data, such as these noted above, help to explain the results obtained in this work. Although root beer is classified as a non-acid soft drink (Jacobs, 1951), it is likely that the acidity is great enough to effect many bacteria. The relatively low pH along with the other factors of carbonation and osmotic pressure serve to eliminate all but a few micro-organisms which may have gained entrance to the drink. Under conditions such as these, it is to be expected that counts would be low. At the higher temperatures there is an increase in the CO_2 pressure, and the effects of the low pH are more detrimental. Therefore, fewer organisms could survive in the soft drink at 34° C than at the lower temperatures.

The second factor operating in creating small microbial populations in the finished product is that soft drink plants observe many sanitary precautions: water must be of unimpeachable sanitary quality and

high bacterial standards are maintained for sugars and syrups (Jacobs, 1951). Repeated bacterial checks are generally performed upon pieces of equipment and upon the beverage during the process of bottling (Insalata, 1956).

Tentative Classification of Representative Bacteria

The tentative classification of the five organisms is most easily reviewed in tabular form. Table VII shows the selections of family and genus for each of the organisms studied and the characteristics which so place them, according to Bergey's Manual of Determinative Bacteriology, 7th edition (1957). Specific classification will be discussed separately.

In some instances, specific classification of the organisms cannot be made with certainty. No attempt is made in the present work to establish new species for those organisms so effected.

Organism "5" has several characteristics which place it with Bacillus pulvifaciens Katznelson, 1950; B. laterosporus Laubach, 1916; B. alvei Cheshire and Cheyne, 1895; and B. circulans Jordan, 1890. These characteristics are sporangia swollen with central to terminal ellipsoidal spores, tendency toward gram variability, no gas production from carbohydrates,

TABLE VII

FAMILY AND GENUS CLASSIFICATION

Organism	Family and Genus	Characteristics
"5"	Bacilliaceae	G+ rod, endospores, carbohydrates fermented with more or less acidity.
	Bacillus	aerobic.
"16"	Bacilliaceae	as for "5".
	Bacillus	as for "5".
"6"	Micrococcaceae	G+, spherical, abundant growth on ordinary media, no visible gas produced by aerobes from carbohydrates.
	Micrococcus	aerobic, irregular masses of cells, occasionally single or pairs, action on glucose oxidative.
"4"	Brevibacteriaceae	G+, long, straight, unbranched rods, motile, no spores.
	no generic placement	
"8"	Brevibacteriaceae	as for "4".
	no generic placement	

and saprophytic ability to grow on ordinary media. Of the four organisms mentioned, B. laterosporus and B. pulvifaciens differ from "5" in several ways, e.g., cultural characteristics. Bacillus alvie differs in many ways also, but it does show one interesting similarity with "5"; the cells often lie side by side. Bacillus circulans is the species which most closely resembles "5". It may be that these two forms are identical since non-motile variations of B. circulans have been observed.

Organism "16" has characteristics which place it with Bacillus megaterium de Bary, 1884. These characteristics include sporangia not swollen by spores, vegetative cell diameter of 0.9 μ or more, and acid production from mannitol. There are two main differences between the two organisms, size and ability to reduce nitrates. Bacillus megaterium is usually 1.3 x 3.0 μ, while "16" is larger, 5.7 x 1.5 μ. Bacillus megaterium does not reduce nitrates; "16" does. The difference in ability to reduce nitrate makes it appear unlikely that the two organisms are identical, although they are probably closely related.

Organism "6", because of its capacity to reduce nitrates to nitrites and its lack of red pigment, is placed with Micrococcus caseolyticus Evans, 1916;

this placement seems to be a matter of coincidence rather than one of taxonomic significance. Micrococcus caseolyticus is described as having "luxuriant" growth, while "6" is fairly conservative in this respect; M. caseolyticus produces acid from mannitol, "6" does not; M. caseolyticus gives an acid reaction in litmus milk and peptonizes it, "6" has a basic reaction and exhibits no peptonization; M. caseolyticus is reported to have a yellow pigment, "6" does not. Furthermore, M. caseolyticus, as its name implies, is associated with dairy products. Therefore, it appears that "6" represents an undescribed species.

The work done with organisms "4" and "8" revealed no significant differences between the two. It was observed that "8" exhibited two types of colonies, but smooth-rough variation in colonial morphology is hardly unusual. It was then assumed that "4" and "8" were members of the same species, "8" being a strain with S-R variation. The organisms will hereafter be referred to as "4".

Organism "4" poses a definite classification problem. Among the well known families with gram positive rods, there is none which can truly claim this bacterium. Table VIII shows major differences between "4" and the pertinent families.

TABLE VIII

SUMMARY OF DIFFERENCES BETWEEN ORGANISM "4" AND SOME COMMON FAMILIES

Families with G- rods	Organism "4"
Lactobacteriaceae: non-motile usually, microaerophilic to anaerobic, poor growth on ordinary media.	motile, facultative aerobe, better growth with O_2, good growth on ordinary media.
Cornybacteriaceae: non motile, irregular forms, granules.	motile, constant rod form, no granules.
Bacilliaceae: endospores	no endospores.

Family Brevibacteriaceae had characteristics which would allow for an organism like "4". These include: gram positive reaction, long unbranched rod forms, no spores, carbohydrates utilized, and motility. The two genera of the family, however, eliminate this rod. Genus Brevibacterium consists of coccoid rods which utilize carbohydrates; organism "4" uses carbohydrates, but it is definitely a large rod. Genus Kurthia contains long unbranched rods which do not use carbohydrates.

In the sixth edition of Bergey's Manual of Determinative Bacteriology (1948) the family Bacteriaceae appears. This family, which has a single genus Bacterium, includes a heterogenous collection of species, gram positive and gram negative rods without endospores, exhibiting complex metabolism. In other words, it includes species "...whose position in the system of classification is not definitely established." This family is in rather sad circumstances since even the type species Bacterium triloculare Ehrenberg, 1828, is not characterized in a way to permit definite identification. It is no wonder that most of the species it contained are re-classified in the seventh edition. The majority are now found in Genus Brevi-bacterium; one organism is placed into Genus Kurthia.

This family (Bacteriaceae), which is no longer recognized, would have accommodated organism "4". It could be suggested that Genus Bacterium be reinstated as one of the genera of Brevibacteriaceae. This genus could then be defined to include gram positive rods, utilising carbohydrated, and, perhaps, reducing nitrates to nitrites. Organism "4" would then have generic classification.

SUMMARY

Studies are made to determine the number and types of micro-organisms ordinarily found in root beer. Effects of varying temperatures and durations of incubation are noted. Representative organisms are characterized and tentatively classified.

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