NUTRITIONAL STUDIES WITH LACTOBACILLUS FERMENTI

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Asparagus juice concentrate and certain other plant products have been observed in this laboratory to be highly stimulatory to *Lactobacillus fermenti*, strain 36, in the presence of an optimal concentration of thiamin, under the general conditions recommended for the assay of thiamin with this bacterium (Sarett and Cheldelin, 1944). A more detailed study, reported here, has shown that growth approximating that induced by asparagus juice concentrate can be obtained on a medium containing an appropriate combination of fructose, maltose, and reducing agents. When this study was near completion, Fang and Butts (1951) reported that either maltose or takadiastase digested starch stimulated growth of *L. fermenti* and thus interfered in the thiamin assay unless the stimulant was included in the basal medium.

EXPERIMENTAL METHODS

Two stock cultures of *L. fermenti*, strain 36 (ATCC 9338), gave very similar results. They were maintained by biweekly transfer on tomato juice agar (Difco) supplemented with 0.05 per cent yeast extract (Difco) and 1 μg per ml of thiamin hydrochloride with incubation for 24 hours at 35 C and storage under refrigeration.

The thiamin assay medium of Sarett and Cheldelin (1944) was used with minor variations as follows: vitamin concentrations (excepting *p*-aminobenzoic acid) increased 10- to 40-fold, magnesium sulfate concentration increased 4-fold, and addition of 10 μg per ml of xanthine, 1 μg per ml of pyridoxamine hydrochloride, 0.04 μg per ml of pyridoxal hydrochloride, 1 μg per ml of nicotinamide, and 12.5 μg per ml of inositol (final concentrations). When used for purposes other than thiamin assay, the medium was supplemented further with excess thiamin (2 μg per ml) and with 0.05 per cent yeast extract (Difco).

Inoculum was grown on autoclaved medium (120 C for 15 min) for 22 to 24 hr at 35 C, centrifuged, resuspended in saline, diluted 1:7, and either added dropwise to 8 ml cultures in 16 mm tubes with a 20 gauge hypodermic needle or pipetted into sterilized, undispensed medium at a 1:150 ratio.

Except as indicated, the media were Seitz filtered to eliminate the effect of heat-reaction products and inoculated before distribution to sterile culture tubes. The test solutions were sterilized similarly in the earlier experiments, but this was omitted later with no apparent loss in reproducibility.

Growth was measured after 16 to 18 hr at 35 C by turbidity readings in a Klett-Summerson colorimeter (with a 660 mμ broad-band filter). When the sample pigment or prolonged heating gave an interfering color, the results were corrected by subtracting the reading of the centrifuged supernate. Except for this the actual readings are reported, the magnitude of the departure of the higher readings from linearity being indicated in figure 1.

The presence of various sugars was established by paper chromatography. The solvent mixture (n-butanol:ethanol:water, 10:1:2, v/v) was allowed to descend for 3 to 6 days. The test solutions included 3,5-dinitrosalicylic acid in NaOH to show all reducing sugars (Jeanes *et al.*, 1951; McCready *et al.*, 1950), resorcinol for ketoses (Partridge, 1948) and aniline for pentoses (Reid, 1950). Paper chromatograms of the glucose, fructose, and ribose used for these studies were consistent with purity of these sugars. A minor secondary spot obtained with maltose could have been either a product of autodecomposition or an impurity.

RESULTS

The growth of *L. fermenti*, strain 36, on *unheated* thiamin assay medium with adequate thiamin was found to be very low unless asparagus juice or certain other materials were added. The dosage-response curve extended from Klett readings of approximately 70 up to 350 with 0 to

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40 mg of asparagus juice solids per ml. Several different lots exhibited similar activity. Citrus molasses, pear molasses, and alfalfa juice were highly active; tomato juice serum, beet molasses, and powdered skimmed milk were somewhat less active. The effect of such extracts was accentuated by the use of a light inoculum and of a short growth period; good growth was obtained on unsupplemented medium with long-continued incubation (about 60 per cent of max at 40 hr).

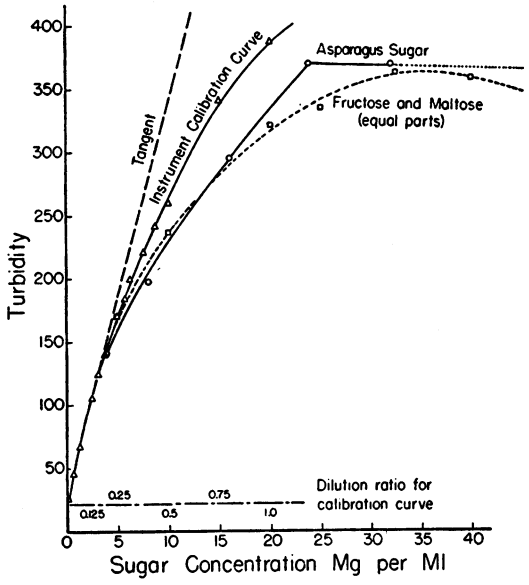

Figure 1. Growth of *Lactobacillus fermenti*, strain 36, (in 17 hr) on asparagus juice medium and on fructose-maltose medium.

Media without glucose but with 0.05 per cent cysteine added.

The instrument calibration curve was obtained by measuring various dilutions (in medium) of a bacterial suspension grown on fructose-maltose medium, and arbitrarily plotting the dilution which read 105 Klett units (containing 1.7×10^8 organisms by plate count) to coincide with 2.5 mg per ml sugar on the horizontal coordinate. The turbidity readings underestimate the extent of growth above approximately 130, as is indicated by the deviation from linearity. The growth curve on fructose-maltose medium, corrected for the nonlinearity of the instrumental readings, would deviate from linearity above readings of approximately 165 (25 per cent of maximum growth).

Figure 1 shows the growth with asparagus juice concentrate with glucose omitted from the medium, as compared with the growth on the most favorable semisynthetic medium (discussed later).

Essentially no stimulation was obtained with additional amounts of the ingredients of the basal medium, with injectable liver extract (Lederle), yeast extract, malt extract, choline, cyanocobalamin, whey, *Lactobacillus bulgaricus* factor concentrate (for which we thank Dr. W. L. Williams of Lederle Laboratories), amino acids, furfuraldehyde, saponin, 2,2′-dithiolisobutyric acid (isolated from asparagus concentrate by Jansen, 1948), and a large number of organic acids, nucleic acid components, and sugars (except as discussed in detail below).

When tested singly, certain sugars stimulated growth and a slight stimulation (5 to 15 per cent of that induced by asparagus juice) was obtained with sodium pyruvate or certain reducing agents. Acetaldehyde was slightly active at 0.025 per cent but was highly toxic at greater concentration.

Attempted concentration of the asparagus factor by means of solvent extractions or adsorption on carbon or ion exchange resins pointed to multiplicity and interdependence of the factors. Whenever fractionation occurred, most of the recoverable activity (25 to 75 per cent of total) followed the sugars, and only a small amount (5 to 25 per cent of total) appeared to separate from the sugars. The activity of separate fractions often totaled less than 50 per cent of that initially present, and recombining the fractions did not always restore the full activity. The fractionated activity appeared to lack stability although the activity of unfractionated asparagus juice was stable to heating at 120 C from pH 2 to 11.

In view of these findings, attention was directed toward combinations of nutrients, such as sugars and reducing agents, which singly had exhibited some degree of activity.

When present as the sole carbon source in unheated media, fructose and maltose gave good growth in 17 hr; glucose, galactose, melibiose, and dextrin gave fair growth; mannose, ribose, raw potato starch, soluble starch, and inulin gave slight growth. Rhamnose, L- or D-xylose, L- or D-arabinose, sucrose, lactose, cellobiose, trehalose, raffinose, melezitose, fructose-1,6-diphosphate, adonitol, dulcitol, mannitol, sorbitol, glycogen, saccharin, salicin, α-methyl-D-glucoside, and aesculin gave no growth.

Of the active carbohydrates, fructose, maltose,

ribose, and dextrin gave additional growth in the presence of an optimal concentration of glucose.

Typical comparisons of growth attained with various levels of glucose, fructose, and maltose added to unheated media initially containing no carbohydrate are shown in figure 2 (no reducing agent) and figure 3 (0.05 per cent cysteine). Cysteine concentrations of 0.025 or 0.075 per cent were only slightly less effective.

Ascorbic acid or sodium thioglycolate produced similar results, but less consistently than cysteine, the effect of ascorbic acid being quite variable. Sodium pyruvate up to 0.04 per cent also had a similar effect; concentrations of 0.08 per cent or higher were toxic. Growth on glucose alone or on a combination of fructose and maltose was stimulated less markedly by the addition of reducing agents than was growth on fructose or maltose alone.

Glucose in excess of 2.5 to 5 mg per ml did not stimulate growth appreciably as the sole sugar and inhibited the response to maltose though not to fructose. In the presence of 0.05 per cent cysteine various proportions of glucose added to 5 or more mg per ml of fructose were used as effectively as an equivalent total amount of sugar as fructose alone. The combination of equal parts of fructose and glucose is illustrated in figure 3. In the absence of cysteine, glucose was relatively ineffective in substituting for the lower concentrations of fructose (figure 2). Fructose in excess of 20 to 25 mg per ml tended to be inhibitory in the presence of reducing agents.

Low levels of maltose (5 mg per ml) generally produced faster growth than like amounts of the other sugars; higher levels were more or less effective than fructose, depending on the level of sugar tested, the presence or absence of cysteine, and the culture conditions of the inoculum (discussed below). The maximum growth on a semisynthetic medium was attained with a combination of fructose and maltose.

Ribose, while able to provide only slight growth alone, was stimulatory when combined with low levels of glucose or fructose but not maltose (table 1). Dextrin was utilized slowly alone, but a combination of equal parts of dextrin, maltose, and fructose was as effective as an equivalent concentration of a mixture of fructose, maltose, dextrin, and ribose. Without the dextrin, however, ribose appeared not to be used when combined with fructose and maltose.

As would be expected, growth with any of the sugars was faster with actively growing inoculum. However, growth on the different sugars was not retarded equally for aged inoculum. Growth on fructose and glucose was as much as 35 per cent poorer with 29 hr inoculum than with 13 hr inoculum. Growth on maltose was reduced still more readily as a result of inoculum maturity. However, the highly favorable fructose-maltose

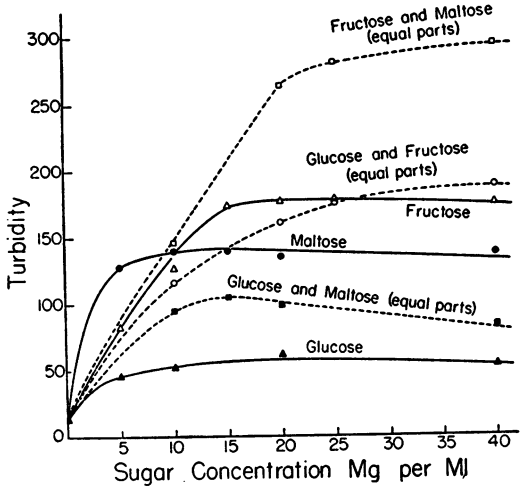

Figure 2. Growth (in 17 hr) with different sugars on media containing no reducing agent.

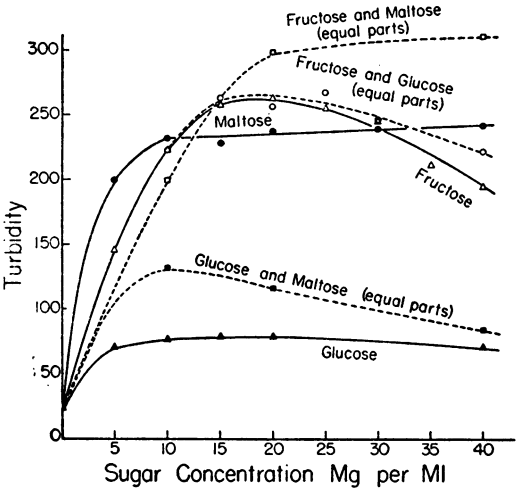

Figure 3. Growth (in 17 hr) with different sugars on media containing 0.05 per cent cysteine.

combination was influenced only slightly, if at all, by the maturity of the inoculum.

The effect of the inoculum was dependent not only on length of incubation of the inoculum but also on the degree of dormancy of the stock stab culture used to inoculate the broth inoculum culture. A 16 hr stock culture gave poorer results than a 24 hr culture. Similarly, still older stock cultures which were stored in the refrigerator for 1 to 3 weeks produced still more active (i.e., physiologically younger) inoculum. Presumably the transfer from less dormant stock cultures initiated more rapid growth, and thus earlier inoculum maturity. These results indicate control of inoculum maturity to be as important as control of amount of inoculum (turbidity) since the resultant growth does not necessarily parallel the turbidity of the inoculum. Variation in the amount of a homogeneous inoculum does, of course, give a corresponding fluctuation in the growth rate.

TABLE 1

Growth on combinations of ribose with glucose, fructose, and maltose (medium containing 0.05 per cent cysteine)

SUGAR CONCENTRATION (MG PER ML)		TURBIDITY	TURBIDITY FOR AN EQUIVALENT AMOUNT OF THE SUGAR INDICATED
None		23*	
Ribose			
2.5		40	
5		37	
10		34	
20		37	
	Glucose		Glucose
2.5	2.5	183	79
5	5	199	80
	Fructose		Fructose
2.5	2.5	189	149
5	5	220	207
10	10	204	277
	Maltose		Maltose
2.5	2.5	166	210
5	5	209	253
10	10	245	269

* Approximately the same reading was given by uninoculated medium.

Heating the assay medium produced stimulatory substances which enabled growth approximately 70 per cent as great as that attained with asparagus juice concentrate in either heated or unheated media. Glucose medium gave typical turbidity readings of 67, 180, and 276, respectively, with no heat, 10 min at 100 C and 12 min at 120 C. Further heating gave no additional increase.

Autoclaving the ingredients of the medium in various combinations showed that glucose was always involved in the production of the stimulants. The greatest effect was produced by autoclaving glucose with the salts solution containing potassium phosphate and sodium acetate. A smaller effect was provided by autoclaving glucose with the casein hydrolyzate. Glucose was activated by autoclaving with sodium hydroxide, sodium acetate, potassium phosphate, or tap water, but not with distilled water. The growth attained on media made up from autoclaved combinations in no case equalled more than 70 per cent of that achieved by autoclaving the complete medium.

TABLE 2

Effect on growth of heating media containing different sugars, with and without cysteine

SUGAR, MG/ML	TURBIDITY					
	No heat		100 C, 10 min		120 C, 15 min	
	No reducing agent	0.05% cysteine added	No reducing agent	0.05% cysteine added	No reducing agent	0.5% cysteine added
Glucose, 25	69	96	107	115	196	200
Fructose, 25	199	317	274	315	255	278
Maltose, 25	136	265	225	265	235	261
Glucose, 12.5, fructose, 12.5	194	310	272	303	259	288
Glucose, 12.5, maltose, 12.5	106	159	139	187	228	270
Fructose, 12.5, maltose, 12.5	303	358	343	362	328	350

Decreased growth was found whenever the cystine of the basal medium (which contained no cysteine) was autoclaved in combinations that did not contain glucose, as described by Dawson and Robson (1950), and ascribed by them to destruction of cystine in the absence of the pro-

tective action of glucose. However, the use of an autoclaved mixture of glucose and cystine was less effective than an unautoclaved mixture.

The beneficial effect of heated glucose medium may be due to production of fructose and a reducing agent (table 2). Data not given here show alkali treated glucose to be stimulatory in media containing fructose and no reducing agent, but of no value when both fructose and a reducing agent (ascorbic acid) were added. The beneficial effect of heat for media containing fructose, maltose, fructose-maltose, and glucose-fructose, but not glucose-maltose, was replaced entirely by cysteine (table 2).

The decrease in pH during growth corresponded closely with the increase in turbidity regardless of which combinations of sugars and reducing agents were used.

Except for the greater departure from linearity in the upper part of the growth curve, the growth response to equal parts of fructose and maltose in a cysteine medium closely paralleled that with an equivalent amount of asparagus sugars (figure 1). This semisynthetic medium does not duplicate closely the sugar composition of asparagus concentrate, however. Paper chromatography showed asparagus sugars (comprising about 77 per cent of the total solids) to be largely glucose and fructose, the fructose spot being somewhat the larger. At least five other slow-moving carbohydrates were evident in small amount. One of these exhibited the same movement rate as maltose; four were ketoses. Pentoses were absent. A determination by Dr. F. Stitt of this Laboratory by a yet unpublished method involving chlorous acid oxidation showed 37 per cent of the sugar to be aldose (thus mainly glucose, according to the chromatography results). Fructose thus is estimated to account for 50 per cent or more of the sugar with the small remaining percentage being unidentified. Optimal growth has not been obtained on a semisynthetic medium containing comparable proportions of glucose and fructose.

Growth on asparagus concentrate was increased only slightly (about 5 per cent) by addition of 5 to 20 mg per ml of a mixture of fructose and maltose, and no significant increase was produced by dextrin and ribose. Addition of 20 mg per ml glucose produced a distinct growth lag.

Paper chromatography was employed to determine whether the organism forms glucose during growth on maltose medium. No glucose could be detected in the centrifuged supernatant from cultures grown in the presence of excess maltose.

DISCUSSION

Attempts to identify the active factors of asparagus juice for *L. fermenti* with any of the recently reported growth stimulants indicate differences in most cases. Metcalf *et al.* (1946) reported a somewhat alkali-labile growth factor in tomato serum and certain other vegetable juices which was active for *L. fermenti*, strain 36, but further comparison is not possible. The heat-stable activity of malt sprouts for *Streptococcus lactis* and *Lactobacillus casei* reported by Colio and Babb (1948) may be similar.

The stimulation provided by asparagus juice concentrate appears to be not vitamin-like in nature, but rather to be due largely to a favorable combination of carbohydrates and reducing agents. While growth with fructose, maltose, and cysteine, or with these ingredients plus ribose and dextrin, has reached approximately the same level as that attained with asparagus juice, the stimulation due to asparagus has not been duplicated. Maximum growth on the semisynthetic medium has been obtained only by inclusion of maltose in proportions exceeding considerably the possible maltose content of asparagus juice. The fructose content of the asparagus juice was proportional to that which was found to be desirable in the semisynthetic medium.

The failure of many lactic acid bacteria to initiate growth promptly on unheated media or media heated separately from glucose has been discussed by Snell *et al.* (1948). As is the case here, reducing agents often have substituted partially or completely for the stimulatory heat-reaction products as has been reported by Rabinowitz and Snell (1947) for *Streptococcus faecalis*, by Rose and Peterson (1949) for three lactics, and by Hoffmann *et al.* (1949) and Stokstad *et al.* (1949) for *Lactobacillus leichmannii*. Hoffmann *et al.* specifically mention the effectiveness of asparagus juice concentrate in supplying such a reducing agent.

Smiley *et al.* (1943) obtained prompt growth of *Streptococcus salivarius* by addition of pyruvic acid or acetaldehyde to media containing separately sterilized glucose. Snell *et al.* (1948) found pyruvic acid highly effective with *Lactobacillus bulgaricus* as a substitute for autoclaved glucose. Our studies, however, have shown very limited effectiveness for these substances.

The use of maltose (glucopyranose-4-α-glucopyranoside) in preference to glucose is similar to other cases cited by Snell *et al.* (1948), who have discussed the energy advantage which an organism might achieve by phosphorolytic cleavage of a disaccharide rather than hydrolysis before utilization. They described a strain of *L. bulgaricus* which used lactose more readily than either glucose or galactose or a mixture of the two. Wright (1936) described a comparable situation for use of lactose and sucrose by *Streptococcus thermophilus*. *Pseudomonas saccharophila*, as reported by Doudoroff (1945), oxidizes raffinose and sucrose (in low concentrations) more rapidly than melibiose or any of the hexose constituents of raffinose separately or together. If supplied in high concentration, melibiose is utilized rapidly while the monosaccharides are still utilized slowly. Prompt fermentation of maltose but not of glucose has been reported by Pelczar and Doetsch (1949) for several strains of *Neisseria* and by Doudoroff *et al.* (1949) for a mutant strain of *Escherichia coli*. Utilization of maltose by a different metabolic pathway from utilization of glucose was suggested for *Neisseria meningitidis* from experiments on growth (Fitting and Scherp, 1951) and on washed cells (Fitting and Scherp, 1952).

Monod and Torriani (1948) have reported the inhibitory action of glucose on an enzyme (amylomaltase) produced by a strain of *E. coli* which converts maltose into glucose and a polysaccharide. The phosphorolysis of sucrose reported by Doudoroff (1943) also is inhibited by glucose. The inhibition of maltose utilization by *L. fermenti* by glucose but not by fructose may be analogous to these cases.

The results presented here might have escaped detection by conventional qualitative fermentation tests, and thus support the suggestion of Snell *et al.* (1948) that quantitative work may indicate that preferential use of oligosaccharides may be relatively common.

The tendency toward decreased use of maltose by more mature cells is unusual and not readily explained by adaptive enzyme formation. The anomaly of better growth at low sugar concentrations on maltose than on a mixture of maltose plus fructose, and on the mixture rather than on maltose alone at higher sugar concentrations, also remains unexplained. The hypothesis that fructose counteracts an inhibitory concentration of glucose produced from the maltose lacks support since no glucose formation was demonstrated in cultures grown on excess maltose.

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The observations of Ramsey and Lankford (Bact. Proc. (1952), p. 165) have come to our attention recently. These authors reported marked stimulation of *L. fermenti*, strain 36, by an autoclaved mixture of glucose plus phosphate, and concentrated the active factor by chromatography. Our growth responses are not inconsistent in view of the fact that we worked with less dilute inoculum and obtained higher maximum growth.

SUMMARY

Asparagus juice concentrate and certain other plant products are highly stimulatory to *Lactobacillus fermenti*, strain 36, on *unheated* medium. The effect is most apparent with a light inoculum and a short incubation period. Approximately 70 per cent of the stimulation may be provided by *heating* the medium. Glucose reaction products (fructose plus reducing agents) simulate the stimulation due to heat.

Replacement of glucose in the medium by maltose, fructose, and cysteine permits growth equalling that obtained with asparagus concentrate although such a medium does not represent a duplication of the composition of the asparagus concentrate. Either maltose or fructose alone permits more rapid growth than does glucose, and the presence of fructose permits more rapid growth on either maltose or glucose. The addition of reducing agents facilitates growth on fructose and maltose most markedly. The condition of the inoculum is also important. The improved growth on maltose, as compared with glucose, and its inhibition by glucose indicate that *L. fermenti*, like numerous other organisms, attacks the oligosaccharide most efficiently by some mechanism other than direct hydrolysis.

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