USDA Attachment D

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USE OF FOOD AND FOOD INGREDIENTS TO PREVENT DIARRHEAL DISEASES IN CHILDREN IN DEVELOPING COUNTRIES

(58-319R-7-021)

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JUSTIFICATION

Acute diarrheal disease is a major cause of morbidity and mortality throughout the world, particularly among children under 5 years of age in developing countries. Approximately 5 to 10 million children in developing countries die each year as a result of diarrheal disease. Peak incidence occurs between the ages of 6 months to 2 years. Diarrheal disease involves continuous loss of fluids and electrolytes which leads to dehydration, maladsorption, and malnutrition. Advanced stages can produce failure in the normal parental defense mechanisms against microbial invasion and can eventually lead to death. In an analysis of previous studies on acute diarrheal disease in developing countries, Snyder (Center for Disease Control) and Merson (World Health Organization) concluded that the mean incidence of diarrhea for children in developing countries under the age of five is 2.2 episodes per child per year. They used 1980 population data to estimate a total yearly morbidity of 744 to 1000 million and a mortality of 4.6 million for children under the age of five in Africa, Asia (excluding China), and Latin America. Although not nearly so critical a problem in developed countries such as the United States, acute diarrheal disease still remains a major source of morbidity, hospitalization, and loss of economic resource. Acute diarrheal disease is ranked second only to the common cold as a cause of time lost from work in this country.

Although enterotoxigenic *Escherichia coli* (ETEC), rotavirus, and *Shigella* have been reported to be most commonly involved in outbreaks of diarrheal disease in developed and developing countries, other microorganisms, such as *Campylobacter jejuni/coli*, may be more commonly involved than previously reported. The primary causative

agents, as well as the incidence, of diarrheal disease varies among different countries as well as across age, sex, and social sub-groups within each population. For example, in Bangladesh, the entero-pathogens most commonly identified among children with diarrheal disease in a treatment center were rotavirus (45%), *E. coli* (28%), *Vibrio cholerae* (8%), and *Shigella* (5%). However, when a field survey was conducted, *E. coli* (20%), *Shigella* (15%), and rotavirus (11%) were most frequently identified as causative agents. Although other bacterial (*Salmonella* and *Yersinia*), viral (Norwalk agent), and parasitic agents (*Giardia*, *Cryptosporodium*, and *Entamoeba histolytica*) have been implicated in studies of diarrheal illness among children, their significance appears to be less than that of *E. coli*, rotavirus, *V. cholerae*, and *Shigella*.

ETEC is one of the most important causative agents of diarrheal disease in human infants in developing countries. *E. coli* commonly occurs in early weaning foods, particularly those contaminated by impure water and unclean utensils or prepared under insanitary conditions. The problem is further aggravated by rapid multiplication of *E. coli* in foods not consumed soon after preparation. Pathogenesis by ETEC involves adherence to epithelial mucosa through adhesive pili attached to the bacterial surface, colonization of the small intestine, and subsequent secretion of enterotoxins.

Milk and colostrum contain immunoglobulins, lysozyme, lactoferrin and lactoperoxidase that are known to possess antibacterial properties Immunoglobulins protect neonates against diarrheal disease caused by ETEC through passive immunity and by preventing adhesion of entero-pathogens to intestinal mucosa. However, milk and colostrum also appear to contain [illegible]

of ETEC to intestinal mucosa. These non-immunoglobulin substances would appear to possess structures similar to pili recepto s on the small intestine brush border membrane. In other studies, human milk appeared to contain various non-immunoglobulin fractions that inhibit heat-labile enterotoxin (LT) produced by ETEC and cholera toxin, as well as prevention of adhesion of V. cholerae (hemagglutination). Gangliosides from human milk inhibit fluid secretion induced by cholera toxin and ETEC LT in the rabbit intestinal loop assay.

Progress made in this study to determine the ability of lactic acid-producing bacteria to prevent colonization by ETEC would be expected to lead to development of new improved treatments to prevent diarrheal disease among human infants. Development of new preventive treatment would be of tremendous value to weanling infants in developing countries.

ABBREVIATIONS

MEDIA

EMB	Levine's Eosin Methylene-blue Agar (O_2, plates) --enumeration of *E. coli*
MRS	MRS Lactobacilli Agar (AnO_2, roll tubes) --enumeration of lactic acid producing bacteria (*Lactobacilli* and *Streptococci*)
RGCA	Rumen fluid-Glucose-Cellibiose Agar (AnO_2, roll tubes) --enumeration of total bacteria

EXPERIMENTAL GROUPS

SC	Suckling, control
WC	Weaned, control
WM	Weaned, Medipharm supplement
WL	Weaned, Lab isolate (2CA)
WE	Weaned, *E. coli* challenge
WME	Weaned, Medipharm supplement, *E. coli* challenge
WLE	Weaned, Lab isolate (2CA), *E. coli* challenge

EXPERIMENTAL SAMPLES

PE	*Pars esophagus*
St	Stomach
SI,S	Small intestine
C	Cecal contents

MISC.

LAB	Lactic Acid Bacteria
EC	*E. coli*

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SUMMARY OF RESULTS

Streptococcus faecium M74 (Pig Trials 1, 2, 3, &5)

1. None of the piglets in the SC (n=12), WC (n=20), or WM (n=21) groups developed clinical symptoms of diarrheal disease during the course of this study. However, eighteen (18) of the twenty-two (22) piglets challenged with an enterotoxigenic strain of E. coli developed clinical symptoms. Only nine (9) of twenty-one (21) piglets receiving three oral doses of S. faecium prior to E. coli challenge were observed to develop diarrhea.

   Thus, prior dosing with S. faecium produced greater than a 50% reduction in incidence of diarrheal disease produced in weanling piglets by enterotoxigenic strains of E. coli.

2. The SC group contained significantly fewer E. coli in their PE and S than did any other group of weanling piglets. The WME group contained elevated levels of E. coli in their PE and C [SF 1].

3. Little differences could be detected in the levels of E. coli detected at various sites in the gastrointestinal sites of WE piglets which developed diarrhea [WE(D)] and those which did not [WE(N)]; only fecal material (F) of WE(N) piglets contained fewer E. coli [SF 2].

4. Piglets which developed diarrhea [WME(D)] in the WME group actually had fewer E. coli in their PE and S than did piglets which did not show clinical symptoms [WME(N)]. Only in C and F did diarrheal pigs have slightly more E. coli [SF 3].

5. Levels of E. coli detected in the PE, S, and C of piglets sacrificed one week after E. coli challenge were usually lower [compare WE* vs WE & WME* vs WME] than piglets in the same groups which were sacrificed earlier in the study [SF 4].

6. The distribution of lactic acid bacteria was very similar between groups at all gastrointestinal sites [SF 5]. No significant trends could be detected in the distribution of lactic acid bacteria among WE and WME piglets which did or did not develop clinical symptoms of diarrhea [SF 6 & SF7].

7. The ratio of LAB/E. coli was significantly lower in the PE of WME piglets than in any other treatment group. This ratio was significantly greater in the S of piglets belonging to the SC group and slightly greater in the C and F [SF A].

8. WE piglets which did not develop clinical symptoms of diarrhea had significantly greater LAB/E. coli ratios only in F. Only minor differences existed at other gastrointestinal sites between WE piglets which did and did not develop diarrhea [SF B]. Among WME piglets, the ratio of LAB/E. coli was greater in PE, S and C among piglets which did develop diarrhea than those which did not; only in F was the reverse true.

9. Electrophoretic analyses of KF+ circular colonies picked from KF Streptococcus agar plates in Piglet Trial #3 indicate that the S. faecium used to dose piglets in the WM and WME group can be recovered from the PE as well as the S and C of piglets in this group. To our knowledge, these results provide one of the first indications that probiotic organisms can become established at sites (PE) within the gastrointestinal tract without prior treatment to disrupt the normal microflora present. Since diarrheal disease detected in Pig Trial 3 was probably not caused by E. coli (no WE pigs developed symptoms), nothing further can be concluded concerning the protective effect especially among piglets with a high proportion of colonies identified as S. faecium.

9. All SF+ picks from Pig Trial #5 are on plates, are ready to be inoculated into broth and processed for electrophoresis. This portion of the study could not be completed on time since the technician hired on this grant resigned in mid-August to accept another position since this grant was being terminated. We will attempt to complete the study with the limited resources and personnel at our disposal since this information would be helpful when attempting to publish the results of this study.

10. None of the results from Pig Trial 3, except those mentioned in #9 above, were used since none of the piglets in the WE group developed diarrhea. A different E. coli strain was used in Piglet Trials 4 & 5.

11. In Pig Trial 2, the average pH of the stomach contents was below 3.5 and varied little among the five treatment groups. The average pH of S, C, and F usually varied between 5.8 and 6.8. The two highest average pH values occurred in the C and F of piglets from SC group.

The average lactate content in S was greater than in either C or F. Little differences were detected among the five treatment groups except in C where tremendously elevated levels were found in piglets from the WE group.

The average Cl^- levels in small intestines were greater than in either C or F. The small intestines from piglets in the SC group contained significantly less Cl^- than piglets from any other group. In C and F, piglets from SC, WM and WME

contained slightly more Cl^-.

Similar results were obtained in Pig Trial 3 but data from that trial were not used. However, since this information was adding little to our understanding and required significant investment of time and resources, these assays were deleted from Trials 4 and 5 to allow picking of KF+ colonies and subsequent electrophoretic evaluation.

Lab Strain 2CA: (Pig Trials 4 and 5)

1. None of the eleven (11) piglets in the weaned control group developed clinical symptoms of diarrhea while two (2) of the fourteen (14) piglets in the Strain 2CA (WL) control group did. Nine (15) of the fifteen (15) piglets challenged with enterotoxigenic E. coli developed diarrhea while only four (4) of twenty (20) piglets dosed with Strain 2CA prior to E. coli challenge did. However, four (4) other piglets in this group developed borderline diarrhea and two of these piglets (70-3 and 71-9) suffered acute diarrheal disease which resulted in their deaths.

2. In general, the highest levels of E. coli found throughout the gastrointestinal tract were in groups of piglets which were challenged with E. coli (WE and WLE) [2CA 1]. The S and C of piglets in groups WC and WL contained the lowest levels.

3. Piglets in group WE, which developed clinical symptoms of diarrheal disease, actually had lower levels of E. coli throughout the gastrointestinal tract than did piglets in this group which did not contract diarrheal disease or which were borderline [2CA 2]. In the WLE group, piglets with borderline diarrhea had the lowest levels of E. coli throughout the gastrointestinal tract. WLE piglets which contracted diarrhea had lower levels of E. coli in PE and S compared to piglets which did not develop clinical symptoms [2CA 3].

4. In general, piglets in groups WE and WLE had lower levels of E. coli throughout their gastrointestinal tracts one week after oral challenge [2CA 4].

5. Little difference could be detected in the levels of lactic acid bacteria between the various treatment groups throughout all gastrointestinal sites examined [2CA 5]. Additionally, little differences could be detected between WE and WLE piglets which did (D) and did not (N) develop symptoms of diarrhea [2CA 6 & 2CA 7].

6. In general, the higher LAB/E. coli occurred in piglets in groups WC and WL in most sites examined in the gastrointestinal tracts of weanling pigs [2CA 8]. Piglets in groups WE which did exhibit clinical symptoms of

diarrheal disease actually contained higher ratios of LAB to E. coli than piglets which did not (2CA B). Additionally, in group WLE, piglets which developed clinical symptoms of diarrhea, as well as those who were borderline, also had higher ratios of LAB to E. coli than those who did not.

7. In piglets thus far examined in Pig Trial #4, we have been unable to identify but a very few lactic acid bacteria obtained from the gastrointestinal tracts of WL and WLE piglets as Strain 2CA. These studies are continuing.

8. All 2CA picks from Pig Trial #5 are on plates, are ready to be inoculated into broth and to be processed for electrophoresis. These assays could not be completed because the lab technician paid on this cooperative agreement terminated her employment in mid-August to accept another position. Although this project has ended, we will attempt to complete the study with the limited resources and personnel at our disposal.

Miscellaneous

1. During this study, several colonies were picked from MRS roll tubes inoculated with dilutions from pig samples of experimental groups SC and WC in Pig Trial #1 and WE in Pig Trial #2. Based upon evaluation of their ability to inhibit growth of enterotoxigenic E. coli by simultaneous assay, deferred inverted agar assay and deferred $CHCl_3$ assay, Strain 2CA, obtained from cecal contents, was determined to be the best potential probiotic organism.

2. Strain 2CA is a gram-positive, short diplo-rod which is catalase negative. Based on characteristics determined in the laboratory, strain 2CA is a lactic acid bacteria.

3. Both strain 2CA and SF^+ were screened for characteristics thought to be desireable for probiotic organisms. Both 2CA and SF^+ Both are faculative anaerobes and tolerant to both acid and bile. SF^+ has a generation time of 29 min in BHI and produces acid to pH 4.75. 2CA has a generation time of 27 min in MRS and produces acid to pH 4.45. In vitro, strain 2CA has greater ability to inhibit growth of E. coli than SF^+. Strain 2CA also has greater ability to bind, in vitro, pig epithelial cells prepared from the esophagus as well as the pars esophagae of the stomach.

CONCLUSION

In the studies involving *Streptococcus faecium* M74, 81.8% of weanling piglets challenged orally with an enterotoxigenic strain of *Escherichia coli* (ETEC) developed clinical symptoms of diarrhea. However, in the group of piglets which received three oral doses of *S. faecium* prior to *E. coli* challenge, only 42.9% showed clinical symptoms. This 47.6% decrease in the incidence of diarrheal disease clearly demonstrates the effectiveness of using lactic acid bacteria, such as *S. faecium* M74, as probiotics to protect weanling piglets against diarrheal disease.

However, these results could not be explained based on the number of *E. coli* and lactic acid bacteria detected at various gastrointestinal sites in the weanling piglets. For example, little differences could be detected in the numbers of *E. coli* detected in ETEC-challenged piglets which did show clinical symptoms of diarrheal disease and those which did not. Moreover, piglets in the group, which were dosed with *S. faecium* prior to ETEC challenge and did not develop diarrhea, actually had fewer *E. coli* in the pars esophagus and small intestine than piglets which did not develop clinical symptoms of diarrheal disease. These results are not difficult to interpret since we know that diarrheal disease is produced by action of ETEC within the small intestines and not by the number of *E. coli* present. Thus, individual piglets can have large numbers of nonenterotoxigenic *E. coli* within their small intestines and never show clinical symptoms; the presence of ETEC within the small intestine is the key to whether or not piglets contract diarrheal disease.

In similar fashion, we have shown that the ability of certain lactic acid bacteria to protect against diarrheal disease results from the presence of those particular organisms rather than the total number of lactic acid bacteria present. For example, the distribution of lactic acid bacteria throughout the gastrointestinal tracts was very similar for all treatment groups. Moreover, the actual ratio of lactic acid bacteria to *E. coli* was greater in *S. faecium* piglets challenged with ETEC which did produce diarrhea than those which did not. Thus, this commonly used parameter failed to demonstrate any protective action for *S. faecium*. However, we were able to show that the *S. faecium* used in this study did colonize the pars esophagus and is present in the small intestine and caecum of dosed piglets. Thus, protection against diarrheal disease is derived not from the total number of lactic acid bacteria in the gastrointestinal tract but from the presence of only selected organisms which are effective (*S. faecium*, for example) against ETEC.

Similar results were obtained with the lab strain (2CA) of lactic acid bacteria used it this study. Among piglets challenged with ETEC, 60% developed clinical symptoms of diarrheal disease. However, among those receiving prior challenge with three doses of 2CA, only 20% of the piglets

developed clinical symptoms while a similar percentage were borderline. Nevertheless, a dramatic decrease in the incidence of diarrheal disease could be demonstrated by treatment with Strain 2CA. In similar fashion to the study with S. faecium, little differences could be detected among treatment groups in the level of lactic acid bacteria detected throughout their gastrointestinal tracts. Additionally, piglets which received Strain 2CA prior to challenge with ETEC but still contracted diarrhea had higher ratios of lactic acid bacteria to E. coli than those which did not. However, due to the absence of a good screening method for Strain 2CA, we have thus far been unable to consistently demonstrate that Strain 2CA is actually colonizing the gastrointestinal tract. Although we feel that the decreased incidence of diarrheal disease is due to oral challenge with Strain 2CA, we have thus far been unable to demonstrate that this effect is due to colonization of Strain 2CA.

RECOMMENDATIONS FOR FUTURE STUDY

1. Both *S. faecium* M74 and Lab Strain 2CA have been shown to have the ability to offer some protection to weanling piglets against diarrheal disease caused by enterotoxigenic strains of *E. coli* (ETEC). Future studies should determine whether these organisms are also effective against other organisms known to cause diarrheal disease among young children in developing countries.

2. Studies should be conducted to determine the most effective protocol to use in administering *S. faecium* M74 and Strain 2CA so that effectiveness against ETEC will be maximized.

3. Studies should be conducted to determine if other lactic acid bacteria, such as Medpharm Pig Strain A10, are more effective in preventing diarrheal disease mediated by ETEC in piglets. If this studies prove successful, they should be extended to include other microorganisms which produce diarrheal disease in young children.

4. Studies should be conducted to determine the mechanism by which *S. faecium* M74 and Strain 2CA are able to reduce the incidence of diarrheal disease mediated by ETEC. These studies should be designed to determine the gastrointestinal site at which these organisms are effective. This information will allow more effective screening of potential probiotic organisms when this study is expanded to include human trials.

Abbreviated Summary of Animal Protocol

1. Piglets were randomly assigned to one of the following treatment groups:

   Suckling Control (SC) - piglets were removed from their sows and placed with a selected sow and allowed to continue nursing throughout the study; no other source of nutrition was provided these piglets.

   Weaned Control (WC) - piglets were weaned and placed together in a separate pen in a portable facility. Normal piglet weaning diet and water were provided ad lib.

   Weaned ETEC (WE) - piglets were weaned and placed together in a separate pen in the isolation facility. Normal piglet weaning diet and water were provided ad lib.

   Weaned, Medpharm (WM) - piglets were weaned and placed together in a separate pen in the isolation facility. Normal piglet weaning diet and water were provided ad lib.

   Weaned, Medpharm, ETEC (WME) - piglets were weaned and placed together in a separate pen in the isolation facility. Normal piglet weaning diet and water were provided ad lib.

   Weaned, Strain 2CA (WL) - piglets were weaned and placed together in a separate pen in the isolation facility. Normal piglet weaning diet and water were provided ad lib.

   Weaned, Strain 2CA, ETEC (WLE) - piglets were weaned and placed together in a separate pen in the isolation facility. Normal piglet weaning diet and water were provided ad lib.

   After weaning, all piglets in treatment groups WM and WME were given an oral challenge of 1 gm of SF Plus dispersed in 5 ml of distilled water (Trials 1-3) or milk replacer (Trial 5). All piglets in treatment groups WL and WLE were given an oral challenge of 1 gm of Strain 2CA ($\sim 10^9$ CFU/gm).

   At the time of weaning, all piglets were examined for clinical symptoms of diarrhea. The day of weaning was designated day (-2).

2. In the AM of the following day (-1), all piglets in treatment groups WM and WME were given an oral challenge of 1 gm of SF Plus dispersed in 5 ml of distilled water (Trials 1-3) or milk replacer (Trial 5). All piglets in treatment

groups WL and WLE were given an oral challenge of 1 gm of Strain 2CA ($\sim 10^{9}$ CFU/gm).

All piglets were examined for clinical symptoms of diarrhea.

3. In the AM of the next day (0), all piglets in treatment groups WM and WME were given an oral challenge of 1 gm of SF Plus dispersed in 5 ml of distilled water (Trials 1-3) or milk replacer (Trial 5). All piglets in treatment groups WL and WLE were given an oral challenge of 1 gm of Strain 2CA ($\sim 10^{9}$ CFU/gm).

   In the afternoon (starting at 3:00 PM), all piglets in treatment groups WE and WME were given an oral challenge of an enterotoxigenic strain (O157:K88ac:H13 in Trials 1-3 and O149:K88:K91:nm in Trial #5) of Escherichia coli in 5 ml of Trypticase Soy Broth ($\sim 10^{11}$ CFU/dose). All piglets in treatment groups WE and WLE were given an oral challenge of an enterotoxigenic strain (O149:K88:K91:nm) of Escherichia coli in 5 ml of Trypticase Soy Broth ($\sim 10^{10}$ CFU/dose). A different strain was used in Trials 4 and 5 due to problems encountered in Trial #3 in producing clinical symptoms of diarrhea in Group WE.

   All piglets were examined for clinical symptoms of diarrhea.

4. On the next day (+1), selected piglets were transported to the laboratory and sacrificed by electrocution. Stomachs were excised and contents were obtained; 1 gm of each was dispersed in 10 ml of sterile distilled water [pH]. Pars esophagus region was excised from each stomach; 3 gm was dispersed in 30 ml of sterile phosphate-buffered saline. The small intestines were excised; 100 gm of each small intestine [S] were homogenized in 100 ml of sterile phosphate-buffered saline (PBS) and serial dilutions were made [microbiological analyses]. Contents of the caeca were obtained; 1 gm [C] was dispersed in 10 ml of sterile PBS and serial dilutions were made [microbiological analyses]. Contents of the large intestine were obtained; 1 gm [F] was dispersed in 10 ml of sterile PBS and serial dilutions were made.

   All piglets were examined at least three times for clinical symptoms of diarrhea.

5. On the next day (+2), selected piglets were transported to the laboratory and sacrificed by electrocution. Stomachs were excised and contents were obtained; 1 gm of each was dispersed in 10 ml of sterile distilled water [pH]. Pars esophagus region was excised from each stomach; 3 gm was dispersed in 30 ml of sterile phosphate-buffered saline. The small intestines were excised; 100 gm of each small intestine [S] were homogenized in 100 ml of sterile phosphate-buffered saline (PBS) and serial dilutions were made

[microbiological analyses]. Contents of the caeca were obtained; 1 gm [C] was dispersed in 10 ml of sterile PBS and serial dilutions were made [microbiological analyses]. Contents of the large intestine were obtained; 1 gm [F] was dispersed in 10 ml of sterile PBS and serial dilutions were made.

All piglets were examined at least three times for clinical symptoms of diarrhea.

6. On the next day (+3), selected piglets were transported to the laboratory and sacrificed by electrocution. Stomachs were excised and contents were obtained; 1 gm of each was dispersed in 10 ml of sterile distilled water [pH]. Pars esophagus region was excised from each stomach; 3 gm was dispersed in 30 ml of sterile phosphate-buffered saline. The small intestines were excised; 100 gm of each small intestine [S] were homogenized in 100 ml of sterile phosphate-buffered saline (PBS) and serial dilutions were made [microbiological analyses]. Contents of the caeca were obtained; 1 gm [C] was dispersed in 10 ml of sterile PBS and serial dilutions were made [microbiological analyses]. Contents of the large intestine were obtained; 1 gm [F] was dispersed in 10 ml of sterile PBS and serial dilutions were made.

   All piglets were examined at least three times for clinical symptoms of diarrhea.

7. On the next day (+4), selected piglets were transported to the laboratory and sacrificed by electrocution. Stomachs were excised and contents were obtained; 1 gm of each was dispersed in 10 ml of sterile distilled water [pH]. Pars esophagus region was excised from each stomach; 3 gm was dispersed in 30 ml of sterile phosphate-buffered saline. The small intestines were excised; 100 gm of each small intestine [S] were homogenized in 100 ml of sterile phosphate-buffered saline (PBS) and serial dilutions were made [microbiological analyses]. Contents of the caeca were obtained; 1 gm [C] was dispersed in 10 ml of sterile PBS and serial dilutions were made [microbiological analyses]. Contents of the large intestine were obtained; 1 gm [F] was dispersed in 10 ml of sterile PBS and serial dilutions were made.

   All piglets were examined at least three times for clinical symptoms of diarrhea.

8. All serial dilutions were used to inoculate Levine's Eosin Methylene-blue Agar (EMB) [enumeration of E. coli], MRS agar (MRS) [enumeration of lactic acid producers: lactobacilli and streptococci], and Rumen Fluid-Glucose-Cellobiose Agar (RCGA) [enumeration of total bacteria]. EMB plates were incubated aerobically at 37C for 24-48 hrs. MRS and RCGA

roll tubes were incubated anaerobically at 37C for 24-48 hrs.

9. In Pig Trial 2, all stomach, small intestine, caecum, and large intestine contents were dispersed immediately in 20 ml of sterile distilled water by vortexing. The pH was determined using an aliquot (3 ml) from each suspension. These aliquots were stored at -20 C for lactic acid determination. Sigma Kit 826-UV was used to prepare standards and to determine lactic acid concentrations [see Protocol for Lactic Acid Determination]. Absorbance at 340 nm was determined using a Perkin Elmer Lambda 3 UV/VIS spectrophotometer. The remainder (~ 18 ml) of each suspension was stored at -20 C for Cl^- determination. After thawing, 0.38 ml of 5 M $NaNO_3$ was added and the Cl^- content was determined using an Orion ion specific electrode.

10. In Pig Trial 5, selected piglets were transported to the laboratory and sacrificed by electrocution. Stomachs were excised and contents were obtained; 1 gm of each was dispersed in 10 ml of sterile distilled water [pH]. Pars esophagus region was excised from each stomach; 3 gm was dispersed in 30 ml of sterile phosphate-buffered saline. The small intestines were excised; 100 gm of each small intestine [S] were homogenized in 100 ml of sterile phosphate-buffered saline (PBS) and serial dilutions were made [microbiological analyses]. Contents of the caeca were obtained; 1 gm [C] was dispersed in 10 ml of sterile PBS and serial dilutions were made [microbiological analyses]. Contents of the large intestine were obtained; 1 gm [F] was dispersed in 10 ml of sterile PBS and serial dilutions were made.

PIG TRIAL #4 - - 2CA (Lab culture)/ETEC 0149
May 12, 1989

2CA was isolated from the cecum of suckling piglet #2 (106-1) during the first pig trial. Lab tests have indicated that it is most likely a Lactobacillus sp..

2CA challenge:	Day 1:	7.7×10^9 cells/pig (log phase, in 10 ml MRS broth)
	Day 2:	1.8×10^9 cells/pig (same)
	Day 3:	4.2×10^9 cells/pig (same)
ETEC challenge:	Day 3:	8.5×10^{10} cells/pig ETEC 0149:K88:K91:nm (in TSB + 10% glycerol, brought to 5 ml with sterile PBS)

PIG TRIAL #5 - - SF+ (Medipharm) / 2CA (Lab culture)/ ETEC 0157
July 14, 1989

2CA was isolated from the cecum of suckling piglet #2 (106-1) during the first pig trial. Lab tests have indicated that it is most likely a Lactobacillus sp..

SF+ challenge:

Day 1: 3.0×10^9 cells/pig
(5 ml in dH_2O w/Land-O-Lakes milk replacer)

Day 2 and 3: Same as above.

2CA challenge:

Day 1: 5.4×10^9 cells/pig
(log phase, in 10 ml MRS broth w/ Land-O-Lakes milk replacer)

Day 2: 1.6×10^{11} cells/pig (same)

Day 3: 1.1×10^{10} cells/pig (same)

ETEC challenge:

Day 3: 1.0×10^{11} cells/pig
ETEC 0157:K88ac (in TSB + 10% glycerol, brought to 5 ml with sterile PBS)

PREPARATION OF SF PLUS FOR PIG CHALLENGE

SF Plus: Probiotic made by Triple "F" Products, Des Moines, Iowa; contains *Streptococcus faecium* M74

Dose: 1 gm of SF Plus in 5 ml dH_2O per pig

1. The appropriate amount of SF Plus was dissolved in dH_2O with stirring.

2. 5 ml of this mixture was drawn into a 10 cc syringe using a needle.

3. The syringe was capped (without the needle) and placed on ice until the time of challenge.

PREPARATION OF *E. coli* FOR PIG CHALLENGE

Two cultures of enterotoxigenic *E. coli* were obtained (3/88) from the Penn State Reference Center:

ETEC 0157:K88ac:H13
ETEC 0149:K91:K88ac:NM

ETEC 0157 was used for pig studies.

Preparation of cells:

1. 7 ml BHI or TS broth was inoculated with ETEC 0157 from a stock plate and incubated aerobically for 18-24h.

2. This culture was then used to inoculate a 4 l flask containing 2 l TSB and a stir bar. (The inoculum was also streaked onto BHI or TS agar to check for contamination).

3. The flask was incubated aerobically at 37°C, with stirring, for ≈ 18h.

4. The cells were harvested by centrifugation in sterile tubes at 8,000xg for 10 min.

5. The cell pellets were suspended to 40x concentration using TSB + 10% glycerol. One ml aliquots were made into sterile cryovials and stored in liquid N_2. (One aliquot was set aside to use for a plate count and to check for contamination).

6. After 48h (and/or 1 week prior to the challenge study), one aliquot was removed form the liquid N_2 and a plate count was performed.

Preparation of challenge dose:

Each dose of ETEC was made to contain 10^{11} viable cells:

1. The appropiate aliquot of ETEC was removed from the cryovial by using a 10 cc syringe w/needle. The volume in the syringe was brought to 5 ml by diluting with sterile PBS.

2. The syringe was then capped (without the needle) and placed on ice until the pig challenge.

LACTIC ACID DETERMINATION OF PIG SAMPLES

Preparation of sample:

2 gm of small intestine contents, cecum contents, and feces were each suspended in 20 ml sterile dH_2O

↓

these samples were vortexed to disperse the contents, and 3 ml aliquot was centrifuged ≈1 min @ ≈1,000xg

↓

2 ml supernatant was added to 4 ml cold 10% TCA (10% TCA = 0.6 N trichloroacetic acid)

↓

this mixture was vortexed for 30 sec. and placed on ice for 5 min.

↓

the sample was filtered using a 0.45 μm chemical-resistant filter and frozen until the time of assay

SIGMA kit 826-UV was used to determine lactic acid concentrations; standards were prepared using the kit and treated same as samples on the day of experiment. (Standards were added to the cold TCA, mixed, filtered, and frozen until the time of assay).

Instructions in the kit were followed with the following exceptions:

* Reaction volumes were all cut in half (total final volume = 1.5 ml).
* Both UV and Vis lamps were used **simultaneously** to read results at Abs 340 nm.

A standard curve was used to determine the concentration of lactic acid. These results were compared to the concentrations that were calculated from the absorbance values of the samples (equations given in kit booklet). The concentrations determined using the standard curve were comparable to those determined by calculation. Although, this kit is intended for determining the amount of lactate in blood, there appears to be no problem in using it to determine the concentrations of lactate in our intestinal samples. However, the presence of blood in any of the samples should be considered as a possible source of error.

The Perkin-Elmer Lambda 3 UV/Vis Spectrophotmeter was used for reading the assay.

SUMMARY OF MICROBIOLOGY (E. coli INHIBITION) ASSAYS ~~RESULTS~~

Two types of assays, simultaneous and deferred, were used to determine if certain tester organisms were able to inhibit growth of an indicator organism. The indicator organism used in our assays was enterotoxic E. coli (O157:K88ac:H13; obtained from the E. coli Reference Center at Penn State). In broth assays, inhibition of the indicator organism can be detected via plate counts and/or direct counts (using a Petroff-Hauser chamber). On agar, inhibition of the indicator organism can be visually detected and the zone of inhibition can be measured.

MRS, BHI, or YCL media were used in the inhibition assays depending on which medium the tester organism grew best. Tester organisms grew best on either MRS, BHI, or YCL, while E. coli grew best on BHI. E. coli cannot grow on MRS, thus MRS was only used as a liquid medium, for growth and maintainence of the tester organisms. YCL agar was often used for the inhibition assays to give the tester organisms as much advantage as possible over the faster growing E. coli indicator.

In the simultaneous assay, the tester and indicator organisms are grown simultaneously in broth or on agar plates. Several factors should be considered when using simultaneous assays: growth rate of the tester and indicator organisms, phase of growth of tester and indicator organisms at time of inoculation, amount of inoculum for tester and indicator organisms, etc. The importance of these factors vary depending

on whether liquid or solid media is used for the assay and what kind of media is used (BHI, YCL, or MRS). The simultaneous assay performed on agar was used most often and is the simplest and quickest inhibition assay.

In the deferred inhibition assay, tester and indicator organisms are grown consecutively. Thus, inhibitory substance(s) released during the growth of the tester organism should be present in the medium at the time of indicator inoculation. Two different procedures ($CHCl_3$ and inverted agar) were used to perform deferred inhibition assays on agar.

The three types of assays we used to determine whether the tester organisms inhibited ETEC were (1) simultaneous inhibition assay, (2) deferred inhibition assay--$CHCl_3$ procedure, and (3) deferred inhibition assay--inverted agar procedure. These assays were all performed on solid medium, either on BHI agar or YCL agar. The protocols for these three assays are included.

SIMULTANEOUS INHIBITION ASSAY

Purpose: To determine the ability of various bacteria (primarily lactic acid producers) to inhibit growth of enterotoxic E. coli (ETEC).

Test and Indicator organisms are grown simultaneously. Thus, inhibition of ETEC may be dependent on growth rate of test organism and early release of diffusable inhibitory substance(s).

Media:

YCL

Yeast extract	0.30 g
Casamino acids	0.50 g
Lactose	1.00 g
KH_2PO_4	0.45 g
Na_2PO_4	0.05 g
	100 ml dH_2O + 1.1 % agar

BHI agar

(YCL agar favors the growth of lactic acid producers over ETEC, while still supporting growth of ETEC; ETEC may get too much of "head start" when using BHI agar--for a more accurate or sensitive assay, use YCL agar)

Bacteria:

Indicator strain = ETEC 0157:K88ac:H13 (obtained from Penn State E. coli Reference Center, 3/88)

Tester strains = Swine isolates of Lactobacillus, Streptococcus, etc.

1. An overnight culture of ETEC was diluted by adding 1 ml to 7 ml BHI broth. 80-100 µl of this dilution was spread to dryness on the appropiate agar plate.

2. One drop (pasteur pipette) of each tester strain was then spotted onto the surface of the agar plate.

3. After drying, one set of plates was incubated aerobically, and the other set incubated anaerobically, at 37°C.

4. Results of inhibition (zones) were read at 6 h and 24 h.

NOTE: Particular attention must be paid to the dryness of the plates to be used. For accurate results, ETEC must be spread to dryness and tester spots should not diffuse!!

References:

Mitchell & Kenworthy. 1976. J. Appl. Bact. 41:163-174.

Aimutis's protocol (Chris Hanson's Labs, Inc.)

DEFERRED INHIBITION ASSAYS

Purpose: To determine the ability of various bacteria (primarily lactic acid producers) to inhibit growth of enterotoxic *E. coli* (ETEC).

Test and Indicator organisms are grown at different times. Thus, inhibitory substance(s) released (at any time) during the growth of the tester organism can diffuse through the agar prior to inoculation with indicator organism.

Bacteria:

Indicator strain = ETEC 0157:K88ac:H13 (obtained from Penn State *E. coli* Reference Center, 3/88)

Tester strains = Swine isolates of Lactobacilli, Streptococci, etc.

$CHCl_3$ Procedure:

Media: BHI agar

NOTE: BHI agar must be prepared in **glass** petri dishes for $CHCl_3$ procedure.

BHI soft agar overlay (7 ml, 0.8% agar)

1. Tester strains were spotted (1 dp using pastuer pipette) onto agar plates, in duplicate. One set of plates was incubated aerobically, the other set was incubated anaerobically, at 37°C for 24-48 h.

2. Tester strains were then exposed to $CHCl_3$ by placing ≈ 7 ml CHCl3 in the lid of the inverted plates (under fume hood).

3. After exposure to $CHCl_3$ for 40 min., the plates were allowed to air dry for 1 h at 37°C (open plates and invert on lids).

4. After the $CHCl_3$ had diffused from the plates, they were overlayed with ≈ 7 ml soft BHI agar (0.8%) that had been seeded with 50 µl of an overnight culture of ETEC (indicator organism).

5. Plates were incubated aerobically and anaerobically at 37°C, as before, and results were read after 6 and 24 h.

References:

Bauernfeind and Burrows (cited by Rolfe et al)

Aimutis's protocol (Chris Hanson's Labs, Inc.)

Agar Inversion Procedure:

Media: BHI agar

(allows for good growth of Lactobacilli and Streptococci, and supports good growth of ETEC; Lactobacilli and Streptococci grow best on MRS agar, however MRS does not support growth of ETEC)

YCL agar

Yeast extract	0.30 g
Casamino acids	0.50 g
Lactose	1.00 g
KH_2PO_4	0.45 g
Na_2PO_4	0.05 g

100 ml dH_2O + 1.1 % agar

(YCL agar favors the growth of lactic acid producers over ETEC, while still supporting growth of ETEC; ETEC may get too much of "head start" when using BHI agar. YCL yields more accurate results in the simultaneous assay and may likewise be more accurate in the deferred-agar inversion assay)

1. Tester strains were spotted (1 dp using pastuer pipette) onto agar plates, in duplicate. One set of plates was incubated aerobically, the other set was incubated anaerobically, at 37°C for 24-48 h.

2. After incubation, the agar was detached from the side of the petri dish using a sterile (flamed) spatula. The plate was then inverted and slapped sharply against the benchtop so the agar fell into the lid of the plate.

3. The exposed surface, previously at the bottom of the dish, was spread with 100 µl diluted ETEC (indicator strain). (Dilution was prepared by transferring 1 ml of an overnight culture of ETEC to 7 ml BHI).

4. Plates were incubated aerobically and anerobically at 37°C, as before, and results were read after 12-24 h.

References:

Kekessy and Piguet. 1970. Appl. Micr. 20:282-283.

Cultures isolated from Pig Trial #1

Following Pig Trial #1, several cultures were isolated from the MRS roll tubes inoculated with the dilutions from the pig samples of experimental groups SC and WC. Simultaneous inhibition assays were performed using these cultures and all but 2 of the 19 tested inhibited EC. These results were quite unexpected! The cultures tested were then compared electrophoretically (Appl. Env. Micro. 39:900-907; Moore et al., 1980) to determine if any of the organisms were similar or identical to one another. The following groups were formed:

Group 1: 18SC*
1SA-2

Group 2: 2SA*
9SA
1SA-1

Group 3: 2CB
9FA
18SA
18SB*
19SB-2
19SD
(3A) 19SB-1 (lost during transfers)

Group 4: 1SC*

Group 5: 2CA*

Group 6: 9FB-1*
(6A) 9FB-2*

Group 7: 19SC*

> Note: The following scheme was used for giving the cultures lab numbers:
>
> example: 1SA-2
> 1 = pig no. (lab no.)
> S = small intestine
> A = first colony picked from pig 1's small intestine
>
> "-2" indicates that 1SA turned out to be a mixed culture, and this is the second colony type.

One representative culture from each group and subgroup was tested for inhibition of EC. The cultures chosen are asterisked. All the organisms isolated from this pig trial were gram positive, and all but 1SC grew best anaerobically using MRS medium. Culture 1SC was the only strict anaerobe and did not grow well on MRS. 1SC was grown on BHI and this was used in all the assays performed on 1SC. On all other cultures, the simultaneous and deferred inverted agar assays were performed on YCL, and the deferred $CHCl_3$ assay on MRS using a BHI overlay.

Results of inhibition studies using cultures isolated from Pig Trial #1

	Assay					
			Deferred			
	Simultaneous		Inv. Agar		$CHCl_3$	
Culture	O_2	AnO_2	O_2	AnO_2	O_2	AnO_2
18SC	+	+	–	+	–	+
2SA	+	+	–	+	–	+
18SB	+	+	–	+	–	+
1SC	–	–	–	–	–	–
2CA	+	+	+	+	+	+
9FB-1	–	+	–	+	–	+/–
9FB-2	+	+	–	+	–	+
19SC	+	+	–	+	–	+

Notes:

<u>Simultaneous assay</u>--

In the aerobic simultaneous assays, the inhibition was always at the spot of tester growth, and , with the exception of 2CA, infrequently extended 1 mm beyond the tester spot. 2CA inhibited up to 4 mm from the tester spot. 1SC did not inhibit EC.

In the anaerobic simultaneous assays, the inhibition was still at the spot of tester growth, and sometimes extended 3 mm out. Again, 1SC did not inhibit.

All MRS (and BHI) controls were negative for inhibition.

<u>Deferred, inverted agar assay</u>--

In the aerobic inverted agar assay, EC was only inhibited by 2CA.

Under anaerobic conditions, however, EC was inhibited for 1-15 mm beyond the edge of tester growth. 1SC was the only culture which did not inhibit EC under anaerobic conditions.

All MRS (and BHI) controls were negative.

Deferred, $CHCl_3$ assay--

Again, only 2CA inhibited EC under aerobic conditions (upto 10 mm beyond tester spot).

Under anaerobic conditions, EC was inhibited by all testers except for 1SC and 9FB-1 (upto 22 mm beyond the spot of tester growth). 1SC did not inhibit EC anaerobically, and 9FB-1 was questionable.

All MRS (and BHI) controls were negative.

Conclusions

2CA is the best inhibitor among the cultures isolated from pig trial #1. Note that this culture was obtained from a cecum sample.

See Conclusions under Inhibition Studies using cultures isolated from Pig Trial #2.

Cultures isolated from Pig Trial #2

Following Pig Trial #2, several colonies were picked from the MRS roll tubes inoculated with dilutions of small intestine from WE pigs 128-1 and 135-7 (lab numbers 8 and 19, respectively). (These two pigs showed fewer clinical symptoms of diarrhea than the other WE pigs sacrificed). Four colonies were picked from pig 128-1 and labelled WE 8-1, WE 8-2, WE 8-3, and WE 8-4. Similarly, the three colonies picked from pig 135-7 were labelled WE 19-1, WE 19-2, and WE 19-3. These cultures were gram stained and tested for their ability to inhibit ETEC 0157 *in vitro*. Characteristics of these cultures are given below:

Culture	Gm. Stain	Shape, arrangement	Col. Morph.	Hemolysis
WE 8-1, WE 8-2	+	very short rods, poss. cocci, short chains	circular	-
WE 8-3, WE 8-4	+	s-med rods, pallisades, chains, gran.	rough-edged	-
WE 19-1	+	rods, chains	rough-edged	no growth
WE 19-2	+	short rods, curved chains	circular	-
WE 19-3	+	cocci, diplo, sm. clusters	circular	α

Based on the above characteristics and electrophoretic comparisons of the seven WE cultures, the cultures were grouped as following:

WE 1: WE 8-1
WE 8-2

WE 2: WE 8-3
WE 8-4

WE 3: WE 19-1

WE 4: WE 19-2

WE 5: WE 19-3

All of these cultures are catalase negative, suggesting that they are *Lactobacillus* or *Streptococci*, and all grow best anaerobically on MRS medium. All simultaneous and deferred inv. agar assays were performed on YCL, and all deferred $CHCl_3$ assays on MRS using BHI overlays.

Results of inhibition studies using cultures isolated from Pig Trial #2

	Assay					
			Deferred			
	Simultaneous		Inv. Agar		$CHCl_3$	
Culture	O_2	AnO_2	O_2	AnO_2	O_2	AnO_2
WE 8-1	+	+	−	+	−	+
WE 8-2	+	+	−	+	−	+
WE 8-3	+	+	−	+	−	+
WE 8-4	+	+	−	+	−	+
WE 19-1	+	+	−	+	−	+
WE 19-2	+	+	−	+	−	+
WE 19-3	+/−	+	−	+	−	+

Notes:

Simultaneous assay--

In the aerobic simultaneous assays, the inhibition was always at the spot of tester growth, and infrequently extended 1 mm out. The MRS broth controls were all negative, indicating that the inhibition was due to the growth of the tester organism.

In the anaerobic simultaneous assays, the inhibition was still at the spot of tester growth, and sometimes extended 2 mm out. Again, MRS broth controls were all negative.

Deferred, inverted agar assay--

In the aerobic inverted agar assay, EC was not inhibited.

Under anaerobic conditions however, EC was inhibited for 3-15 mm beyond the edge of tester growth. MRS controls were all negative.

Deferred, $CHCl_3$ assay--

Again, EC was not inhibited under aerobic conditions.

Under anaerobic conditions, EC was inhibited 5-30 mm beyond the edge of tester growth. Controls were all negative.

Conclusions

These results indicate that the tester organisms are producing some substance which is inhibiting the growth of EC. In the aerobic simultaneous assay, inhibition is observed at the spot of the tester organism due to the presence of this substance and its direct contact with the EC. Under anaerobic conditions, the tester organisms continue to produce this substance which may diffuse from the spot of growth, thus the EC may be inhibited beyond the spot of tester growth. Remember that in this assay, the EC are spread on the plate just before the tester organism is spotted. Thus, the tester and EC are growing simultaneously. In the deferred assays, the testers are grown for 18-24h before EC is spread on the plates. Any substance(s) produced by the testers have time to diffuse into the agar. In both the inverted agar and $CHCl_3$ assays, EC was inhibited under anaerobic conditions only, and to a significantly greater extent than in the simultaneous assay. In both deferred assays, the EC is in less contact with the tester spot. This, coupled with the fact that the testers grow less well under aerobic conditions, may explain why inhibition of EC is not seen under aerobic conditions in the deferred assays. Another possibility may be that the substance(s) produced by the tester organisms are not produced under aerobic conditions. It is unlikely that the substance(s) are sensitive to O_2 since inhibition is still observed in the aerobic simultaneous assay.

COMPARISON OF SF+ AND 2CA

Introduction:

Streptococcus faecium M74 is the bacteria used in the probiotic SF plus, which is produced by Medipharm (Triple "F" Products, Des Moines, Iowa). This organism grows best in BHI broth.

2CA was isolated by our lab from the cecum of a suckling pig approximately 3-4 wk old. It is a gram positive, short diplo-rod, and is catalase negative. Based on these and other characteristics, we have determined that 2CA is a lactic acid bacteria. This organism grows best in MRS broth.

LAB isolated by our lab are screened for the desired characteristics of probiotics. The results are then compared to those given by SF+ which is already commercially available.

Characteristic		SF+	2CA
Facultative anaerobe		+	+
Bile & acid tolerant		+[a]	+[b]
Generation time (min)[c]		29 (BHI)	27 (MRS)
Acid production		pH 4.75 (BHI)	pH 4.45 (MRS)
ETEC inhibition *in vitro*[d]		+	+++
Adherence to pig epithelial cells			
In vitro	esophagus	40	75
	pars esoph.	11	65
In vivo	esophagus	N/D	N/D
	pars esoph.	N/D	N/D
Prevention of ETEC diarrhea		?	?

[a]Originally isolated from human intestines.
[b]Originally isolated from cecum of 3-4 wk old suckling pig.
[c]ETEC 0157 has a generation of 22 min (BHI).
[d]See table.
[e]Numbers given are average number of bacteria adhering per epithelial cell.
N/D = not done.

Results of *In Vitro* ETEC Inhibition

Tester organism	Inhibition Assay	Results
2CA	Simultaneous (YCL)	+ (1- 4 mm) O_2, AnO_2
	Deferred inversion (YCL)	+ (5-15 mm) O_2, AnO_2
	Deferred $CHCl_3$ (MRS)	+ (7-21 mm) O_2, AnO_2
SF+	Simultaneous (YCL)	+ (0 mm) AnO_2
	Deferred inversion (YCL)	+ (0- 1 mm) O_2, AnO_2
	Deferred $CHCl_3$ (BHI)	+ (2-15 mm) O_2, AnO_2

PREPARATION OF BACTERIAL CULTURES FOR PAGE COMPARISONS

Gram positive cultures isolated from experimental pigs were prepared for electrophoresis as follows:

Cultures were grown in 7 ml MRS broth + 0.1% $CaCO_3$*
for 12-24h

↓

Cells were harvested by centrifugation at
8,000xg for 10 min.

↓

Cell pellets were suspended in 0.15 ml
0.15 M <u>fresh</u> Tris·HCl, pH 7.0 and a spatula tip
of glass beads (≈15 μm diam.) was added to the cell pellet

↓

This mixture was vortexed for 4 min. to lyse the cells,
and heated @ 55°C for 5 min.*

↓

The mixture was transferred to a microcentrifuge tube
and centrifuged ≈1 min. to pellet the glass beads

↓

The cell lysate was removed from the pellet and
prepared as indicated for electrophoresis:

60 μl sample lysate
5 μl bromphenol blue tracking dye
8-10 μl 60% sucrose

30-40 μl was loaded onto the gel

*These steps help to prevent smearing in the lanes, esp. for gram negative bacterial cultures.

Electrophoresis:

30-40 µl of sample was loaded onto gel (25 tooth comb used)

Run gel for ≈2 h @ 150-200 V (constant)
(lower voltage = less smearing)

Fix gel with 12% trichloroacetic acid (TCA; careful!!) for 20 min. at room temp.

Stain with 0.08% Coomassie blue in MeOH:AcAc:dH_2O (9:4:18) for 10 min. at room temp.

Destain by diffusion using 10% Acetic acid

(staining and destaining times may be adjusted for better contrast)

Reference: Appl. Env. Micro. 39:900-907; Moore et al., 1980.

PREPARATION OF PAGE GEL FOR ELECTROPHORETIC COMPARISON OF BACTERIAL CULTURES

Recipes:

SAGE 1

0.36 g TEMED
21.8 g Tris

dissolve in ≈80 ml dH_2O; pH to 8.9 if necessary; bring volume up to 120 ml with dH_2O

SAGE 2

40.0 g acrylamide (careful!!-neurotoxin!)
2.75 g bis-acrylamide (" ")

dissolve in ≈70 ml dH_2O; bring volume to 100 ml with dH_2O

SAGE 3

0.14 g ammonium persulfate

dissolve in 100 ml dH_2O

SAGE 4

6.25 g acrylamide (careful!!-neuortoxin!)
0.5 g bis-acrylamide (" ")

dissolve in ≈16 ml dH_2O; bring volume to 25 ml with dH_2O

Running gel:

SAGE 1	6.0 ml
SAGE 2	5.1 ml
SAGE 3	6.45 ml
dH_2O	6.45 ml

Stacking gel:

SAGE 1	1.5 ml
SAGE 3	3.0 ml
SAGE 4	1.5 ml

DESIRABLE CHARACTERISTICS

Gram positive, catalase negative
Facultative anaerobe
Bile and acid tolerant
Short generation time (30-60 min.)
Good acid production (pH < 4.5)
Inhibit *E. coli in vitro*
Adhere to epithelial cells (*in vitro*, *in vivo*)
Reduce incidence of *E. coli* diarrhea

SUMMARY OF ISOLATION AND SCREENING OF LAB

Initial isolation and screening

gram stain, catalase test, faculatative anaerobes

Grouping and further screening

electrophoretic analysis
growth and pH curves (generation time, acid production)
ETEC inhibition assays
adherence assays (*in vitro*, *in vivo*)

Challenge trial to determine potential for probiotic use

Isolation and Screening of LAB for Probiotic Potential

Isolation and initial screening:

(1) Pick LAB-like colonies from MRS roll tubes (inoculated with dilutions from pig esophagus, stomach--pars esophagus, small intestine, or cecum); transfer to AnO_2 Chopped-Meat Broth for stock culture

(2) Streak onto Sheep BAP, MRS, BHI, KF Streptococcus, Tomato juice, and Bile Esculin Agar (O_2 and AnO_2);
Inoculate O_2 BHI and MRS broth;
Incubate 24-72 h at 37°C and observe growth characteristics;
Gram stain (note morphology);
Check for catalse activity

(3) Those cultures which behave like LAB on the various media, can grow aerobically (facultative anaerobes), and are gram positive and catalase negative, are used for further screening. The stock culture is grown in MRS broth and several 1-1.5 ml aliquots (+ 4-6 drops sterile glycerol) are frozen and stored in liquid N_2.

Grouping and further screening of LAB isolates:

(1) The isolates to be used for further screening are compared electrophorectically and grouped. Those isolates which give identical electrophoretic patterns are grouped and one representative from each group is used for the following assays.

(2) Growth curves and pH curves are performed in MRS broth for each culture (BHI broth is used only if poor growth is achieved in MRS).

(3) Simultaneous and deferred inhibition assays are performed using each isolate to determine if that isolate can inhibit the growth of ETEC *in vitro*.

(4) *In vitro* adherence assays are performed using squamous epithelial cells scraped from pig esophagus and *pars esophagus*. (These pigs are given Penicillin V to "clean out" their normal flora). The epithelial cells are incubated with the various LAB isloates, and adherence is observed and quantitated using a phase contrast microscope.

(5) *In vivo* adherence assays are performed using pigs "cleaned" with penicillin, and subsequently fed a LAB isolate. (1) Tissue samples from the esophagus and *pars esophagus* are thin-sectioned using a cryostat-microtome, gram stained, and observed using a light microscope (note morphology of bacteria). (2) Microbiology assays of these samples are performed by plating dilutions of the homogenized tissue on MRS agar. Colonies are electrophoretically compared to the LAB isolate fed to the pig to determine if adherence occured.

Challenge trial to determine probiotic potential of LAB isolates:

LAB isolates with the most desired characteristics of a probiotic culture are used in a challenge trial to determine their effectiveness in protecting pigs against *E. coli*-associated diarrhea.

NOTES: (1) Specific antibodies against LAB strains used for challenges would prove useful. Immunoassays such as indirect immunofluorescent assays could indicate presence of the LAB in thin-sections of tissue samples, and help indentify recovered colonies from dilutions of tissue samples. (2) Gram positive bacteria isolated from penicillin-treated pigs (other than those bacteria used in the studies) should be screened for their probiotic potential (growth curves, ETEC inhibition assays, etc.). These penicillin-resistant bacteria would be easy to follow in challenge trials. It is questionable whether the development of antibiotic-resistant strains or the isolation of antibiotic-resistant mutants would be worth our time and effort, but we should take advantage of those bacteria which are isolated during the *in vivo* adherence studies and appear to be penicllin-resistant.

In vitro Adherence Assay

The ability of LAB to adhere to squamous epithelial cells may be an important factor in determining whether or not these bacteria are able to colonize the pig esophagus or stomach (*pars esophagus*). Bacteria which colonize these regions are constantly shed, thus the small intestine, cecum, and colon recieve a continuous supply of these organisms. The ability of certain beneficial bacteria to colonize this area may prove important in maintaining the over all health of the animal by preventing intestinal disturbances caused by pathogenic and opportunistic bacteria.

Various lactic acid bacteria may be tested for their ability to adhere to squamous epithelial cells scraped from the pig esophagus and *pars esophagus* using the following methods.

Day 1: Transfer pig(s) to clean, disinfected cage without feed
Give penicillin water (600U penicillin V/ml H_2O) for 24 h

Day 2: Sacrifice pig(s):

* pH stomach contents, if possible
* collect tissue samples from esophagus and *pars esophagus*
* scrape epithelial cells for *in vitro* adherence assay
* perform microbiological determinations by plating dilutions of homogenized tissue samples
* section tissue samples using cryostat-microtome, gram stain and observe sections using light microscopy for adherence of bacteria to epithelial cells (note morphology of bacteria)

NOTES: Gram positive bacteria isolated from penicillin-treated pigs (other than those which are fed to the pigs) should be screened for their probiotic potential (growth curves, ETEC inhibition assays, etc.). These penicillin-resistant bacteria would be easy to follow in challenge trials.

In vivo Adherence Assay

It is realized that *in vitro* adherence studies are not always good indicators for *in vivo* adherence (Pederson and Tannock, 1989). Therefore, *in vivo* studies are necessary to show that an organism can adhere to epithelial cells *in vivo* and possibly colonize the host. The following methods were used to perform these studies.

Day 1: Transfer pig(s) to clean, disinfected cage without feed
Give penicillin water (600U penicillin V/ml H_2O) for 24 h

Day 2: Feed 10 ml of log phase LAB culture (in MRS) by syringe
Hold feed and H_2O for 24 h

Day 3: Sacrifice pig(s):

* pH stomach contents, if possible
* collect tissue samples from esophagus, and *pars esophagus*
* perform microbiological determinations by plating dilutions of homogenized tissue samples and compare electrophoretic patterns of recovered colonies to LAB test culture
* section tissue samples using cryostat-microtome, gram stain and observe sections using light microscopy for adherence of bacteria to epithelial cells (note morphology of bacteria)

*** *** This schedule may need to be changed *** ***

NOTES: (1) An indirect immunofluorescent assay would be useful in showing adherence of specific LAB to epithelial surfaces of thin-sections.
(2) Gram positive bacteria isolated from penicillin-treated pigs (other than those which are fed to the pigs) should be screened for their probiotic potential (growth curves, ETEC inhibition assays, etc.). These penicillin-resistant bacteria would be easy to follow in challenge trials.

Summary of Observations of Clinical Manifestations of Diarrhea in Suckling Control (SC) piglets during Medipharm Study (Trials 1, 2, and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL
106-1	-	-	-	-*				
104-2	-	-	-	-*				
107-2	-	-	-	-	-*			
101-7	-	-	-	-	-*			
107-15	-	-	-	-	-			
125-2	-	-	-	-	-	-		
125-9	-	-	-	-*				
135-5	-	-	-	-	-*			
126-10	-	-	-	-	-	-		
126-7	-	-	-	-*				(2+/-)
128-5	-	-	-	-	-	-		
135-10	-	-	-	-	-*			
	0/12	0/12	0/12	0/12	0/8	0/3		0/12

Summary of Observations of Clinical Manifestations of Diarrhea in Weanling Control (WC) piglets during Medipharm Study (Trials 1, 2, and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL
106-8	-	-	-	-*				
107-7	-	-	-	-*				
101-8	-	-	-	-	-*			
104-9	-	-	-	-	-*			
99-10	-	-	-	-	-			
125-6	-	-	-	-*				
125-7	-	-	-	-	+/- (3.0)*			
135-9	-	-	-	-*				
135-2	-	-	-	-	-	-		
126-5	-	-	-	-	-	-		
126-3	-	-	-	-	-	-		
128-8	-	-	-	-	-	-		
128-2	-	-	-	-	-*			
71-9	+/- (3.0)	-	-	-	-*			
72-3	-	-	-	-	-	-	-	
74-7	-	-	-	-	-*			
76-5	-	-	-	-	-	-*		
77-6	-	-	-	-*				
78-6	-	-	-	-	-	-	-	
79-3	-	-	-	-	-	-	-	
	0/20 (1+/-)	0/20	0/20	0/20	0/15 (1+/-)	0/8	0/3	0/20 (2+/-)

Summary of Observations of Clinical Manifestations of Diarrhea in

E. coli (WE) piglets during Medipharm Study (Trials 1, 2, and 5). [4.0 (−), normal, firm stool; 3.0 (+/−), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	−2	−1	0	+1	+2	+3	+4	TOTAL
104-3	−	−	−	+(2.0) *				
96-12	−	−	−	+(2.0) *				
101-5	−	−	−	+(1.5)	+(2.5)			
106-3	−	−	−	+(2.5)	+(2.0) *x			
104-4	−	−	−	−	+(2.0) *			
106-10	−	−	−	−	+(2.0)			
107-8	−	−	−	−	+(1.5)			
125-5	−	−	−	−	−	−		
125-10	−	−	−	−	−	+(1.5)		
135-7	−	−	−	−	−*			
126-2	−	−	−	+(2.0) *				
126-9	−	−	−	−	−	−		
126-8	−	−	−	−	+(2.0) *			
128-1	−	−	−	+/− (3.0)*				
128-6	−	−	−	−	−	+(1.5)		
71-3	−	−	−	+(2.5) *				
71-8	−	−	−	+(2.0)	+(2.0)	+(2.0)	−	
77-4	−	−	−	−	−	+(1.5)		
PIG#	−2	−1	0	+1	+2	+3	+4	TOTAL

78-4	-	-	-	+(2.0) *				
75-2	-	-	-	+(1.5)	-	-	-	
77-3	-	-	-	-	-	-*		
75-7	-	-	-	+(2.5)	+(1.5) *			
	0/22	0/22	0/22	11/22	8/16	4/8	0/3	18/22

Sumary of Observations of Clinical Manifestations of Diarrhea in S. faecium (WM) piglets during Medipharm Study (Trials 1, 2, and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL
104-1	-	-	-	-*				
102-8	-	-	-	-*				
106-6	-	-	-	-	-*			
107-6	-	-	-	-	-*			
106-4	-	-	-	-	-			
107-4	-	-	-	-	-			
135-1	-	-	-	-	-	-		
135-6	-	-	-	-	-	-		
126-1	-	-	-	-	-*			
126-11	-	-	-	-	-	-		
126-4	-	-	-	-	-	-		
128-3	-	-	-	-*				
128-4	-	-	-	-	-*			
128-9	-	-	-	-*				
71-7	-	-	-	-	-	-*		
70-4	-	-	-	-	-	-*		
73-4	-	-	-	-*				
74-8	-	-	-	-	-	-	-	
75-4	-	-	-	-	-	-	-	
78-3	-	-	-	-	-	-	-	
78-9	-	-	-	-	-*			
	0/21	0/21	0/21	0/21	0/16	0/9	0/3	0/21

Sumary of Observations of Clinical Manifestations of Diarrhea in

S. faecium piglets challenged with E. coli (WME) during Medipharm Study (Trials 1, 2, and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL
106-11	-	-	-	-*				
107-10	-	-	-	+(2.0) *				
101-6	-	-	-	-	-*			
101-2	-	-	-	-	+/- (3.0)*			
107-3	-	-	-	-	-*			
101-9	-	-	-	-	-			
106-7	-	-	-	-	+(2.5)			
125-4	-	-	-	+/- (3.0)*				
125-3	-	-	-	-	-	-		
125-8	-	-	-	-	+(1.5) *			
135-3	-	-	-	-	-	-		
135-4	-	-	-	+(2.0) *				
126-13	-	-	-	-	-*			
128-7	-	-	-	-	+(2.5)	+(2.0)		
75-6	-	-	-	-	-	-	-	
71-4	-	-	-	-*				
78-1	-	-	-	+(1.5)	+(2.5)			
73-5	-	-	-	-	-*			
74-10	-	-	-	-*				
PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL

77-1	-	-	-	-	-*			
78-7	-	-	-	-	-	+(1.0) *		
	0/21	0/21	0/21	4/21	5/18 (1+/-)	2/7	0/3	9/21 (1+/-)

Distribution of *Escherichia coli* in the gastrointestinal tract of piglets during Medipharm Study (Trials 1, 2, and 5).

GROUP	CFU/g PE	S	C	F
SC		2.6×10^3	7.0×10^7	3.1×10^7
WC	2.2×10^6	2.0×10^6	3.4×10^8	3.5×10^8
WE	1.3×10^5	3.7×10^6	1.3×10^8	6.3×10^7
WE(D)	1.3×10^5	4.1×10^6	9.2×10^7	1.1×10^8
WE(N)	1.3×10^5	1.8×10^6	6.1×10^7	3.0×10^6
WM	2.1×10^4	3.3×10^5	6.6×10^7	1.8×10^8
WME	9.6×10^7	1.7×10^6	1.6×10^9	3.2×10^8
WME(B)		2.9×10^5	1.7×10^8	1.0×10^8
WME(D)	6.7×10^4	4.1×10^5	3.7×10^9	8.5×10^8
WME(N)	1.3×10^8	2.6×10^6	3.9×10^8	7.1×10^8
WC*	5.6×10^3	6.7×10^4	1.2×10^7	
WE*	4.9×10^5	5.3×10^5	6.2×10^7	
WE(D)*	4.9×10^5	5.3×10^5	6.2×10^7	
WME*	9.1×10^4	2.1×10^5	4.4×10^8	
WME(D)*	5.2×10^4	1.5×10^5	1.1×10^8	
WME(N)*	1.7×10^5	3.2×10^5	1.1×10^9	

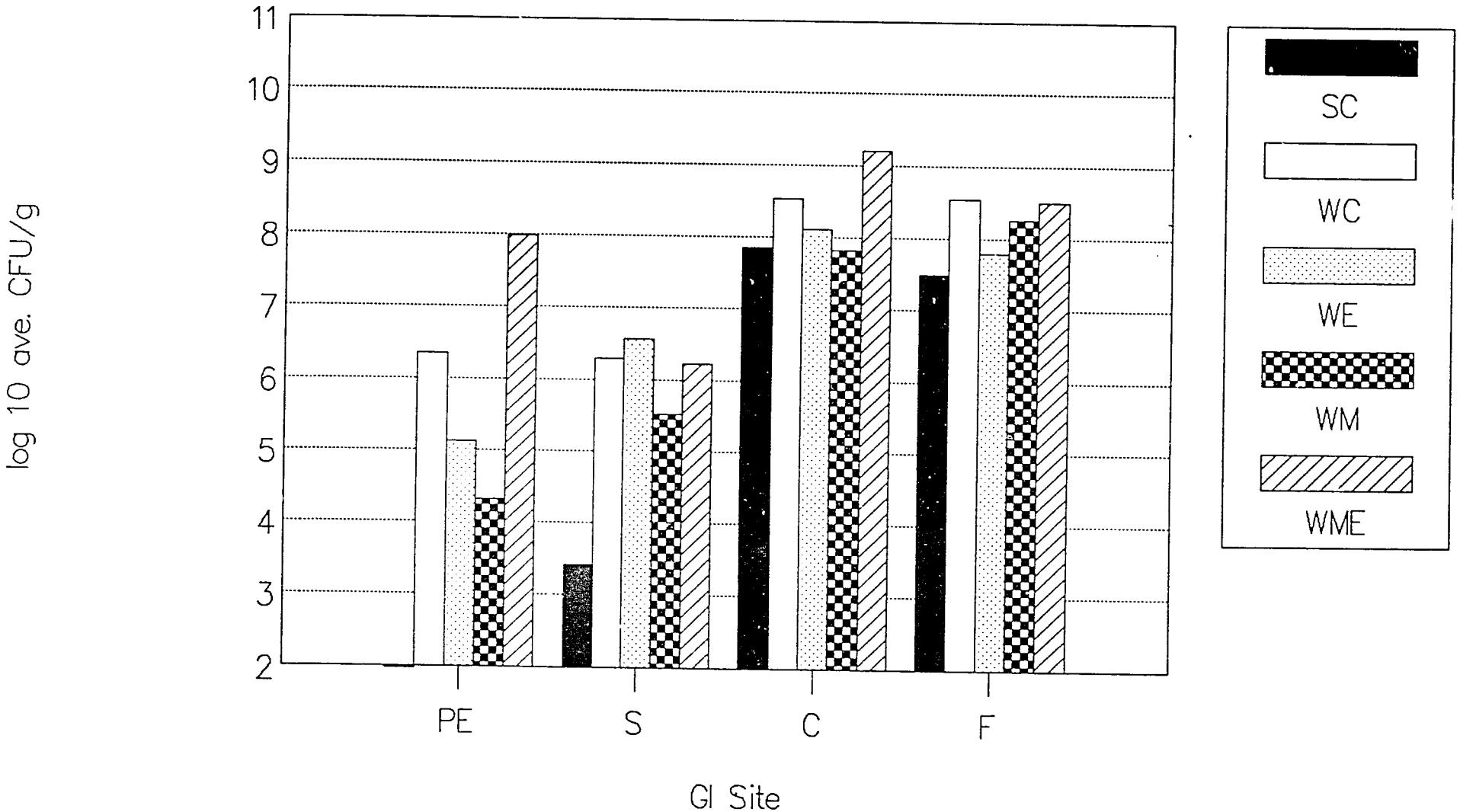
Distribution of lactic acid bacteria in the gastrointestinal tract of piglets during Medipharm Study (Trials 1, 2, and 5).

GROUP	CFU/g PE	S	C	F
SC		1.5×10^8	3.6×10^9	5.8×10^9
WC	3.1×10^8	3.7×10^3	2.5×10^9	7.4×10^9
WE	1.3×10^8	2.0×10^8	1.7×10^9	2.1×10^9
WE(D)	1.1×10^8	2.3×10^8	1.7×10^9	2.2×10^9
WE(N)	1.7×10^8	5.0×10^7	1.9×10^9	9.9×10^8
WM	1.5×10^8	1.5×10^8	1.5×10^9	2.8×10^9
WME	1.6×10^8	1.2×10^8	6.3×10^9	2.0×10^{10}
WME(B)		1.0×10^7	1.6×10^8	8.8×10^7
WME(D)	4.3×10^7	1.6×10^8	1.8×10^{10}	7.2×10^8
WME(N)	2.0×10^8	1.1×10^8	5.7×10^8	4.7×10^{10}
WC*	2.6×10^8	1.1×10^7	5.2×10^8	

Distribution of anaerobic bacteria in the gastrointestinal tract of piglets during Medipharm Study (Trials 1, 2, and 5).

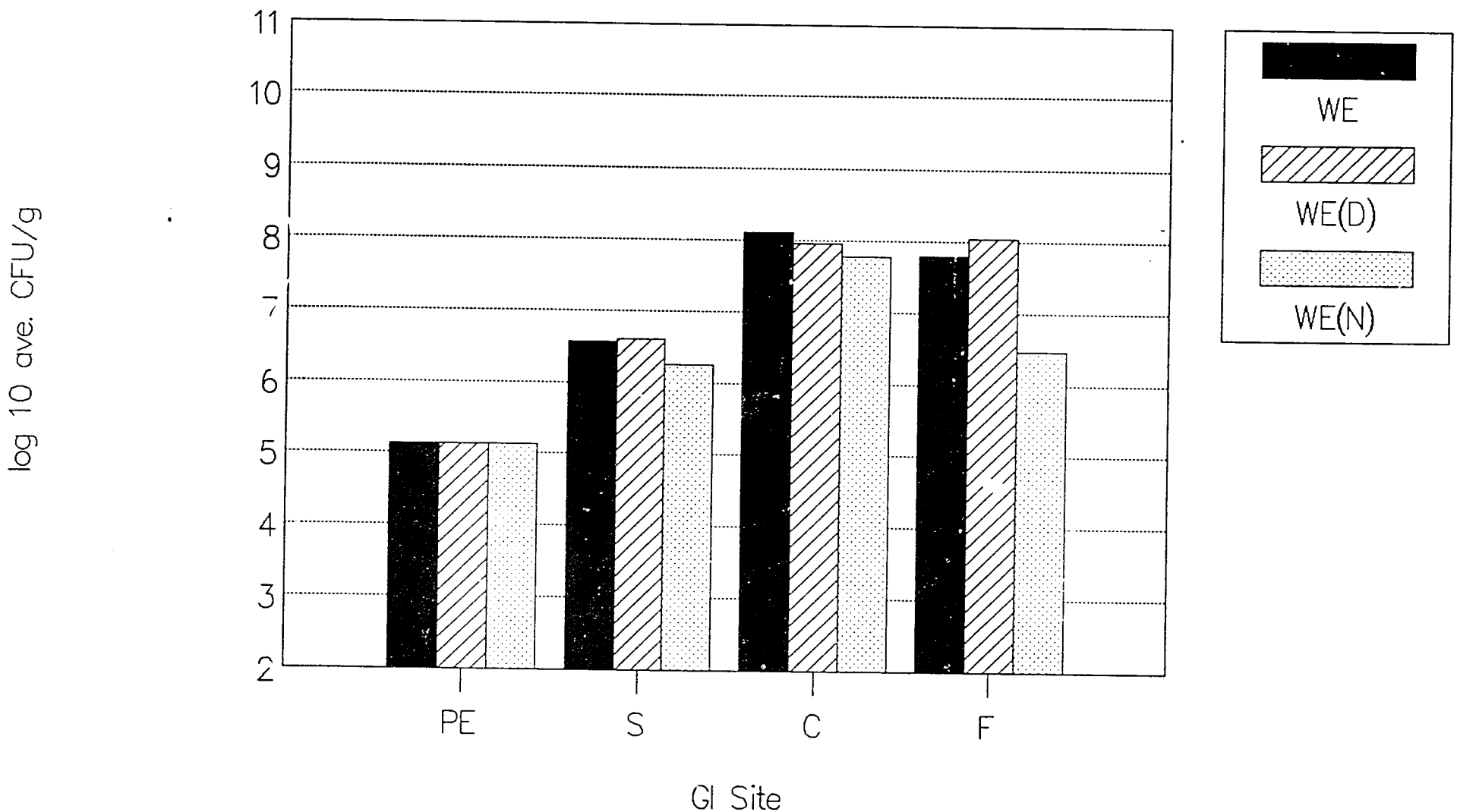
GROUP	CFU/g PE	S	C	F
SC		6.3×10^{7}	1.3×10^{9}	2.1×10^{10}
WC	6.9×10^{8}	1.2×10^{8}	2.5×10^{9}	3.6×10^{9}
WE	2.2×10^{8}	5.8×10^{8}	6.3×10^{9}	4.7×10^{9}
WE(D)	1.2×10^{8}	6.7×10^{8}	7.3×10^{9}	5.2×10^{9}
WE(N)	5.3×10^{8}	8.9×10^{7}	9.7×10^{8}	8.4×10^{8}
WM	1.1×10^{8}	1.1×10^{8}	2.6×10^{9}	2.1×10^{9}
WME	3.5×10^{8}	1.6×10^{8}	2.7×10^{9}	1.7×10^{9}
WME(B)		1.2×10^{7}	3.5×10^{9}	5.8×10^{8}
WME(D)	5.6×10^{7}	3.7×10^{8}	5.5×10^{9}	9.2×10^{8}
WME(N)	4.4×10^{8}	6.5×10^{7}	9.8×10^{8}	2.6×10^{9}

Presence of E. coli in Experimental Groups



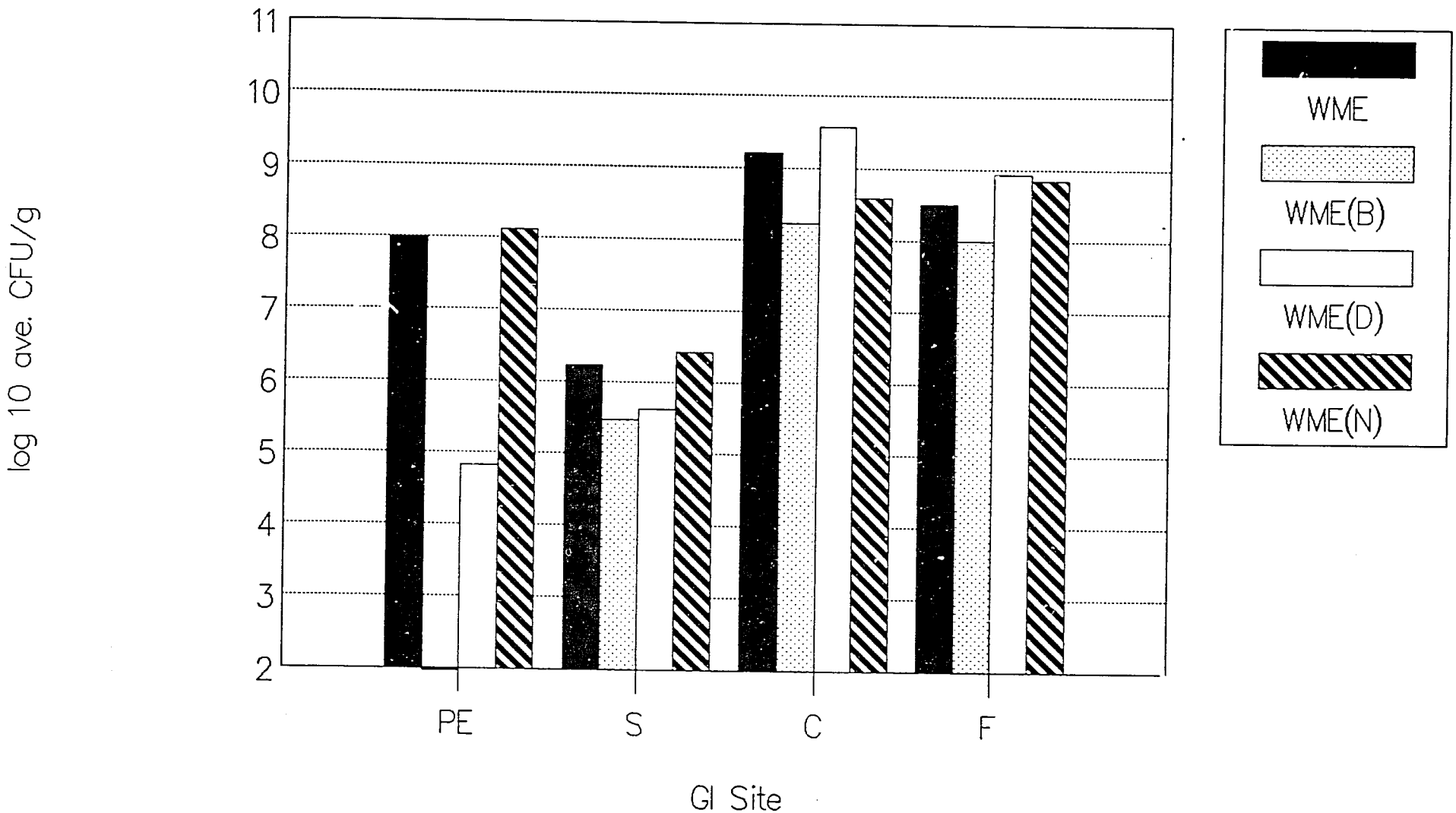
SF 1

Presence of E. coli in Experimental Groups



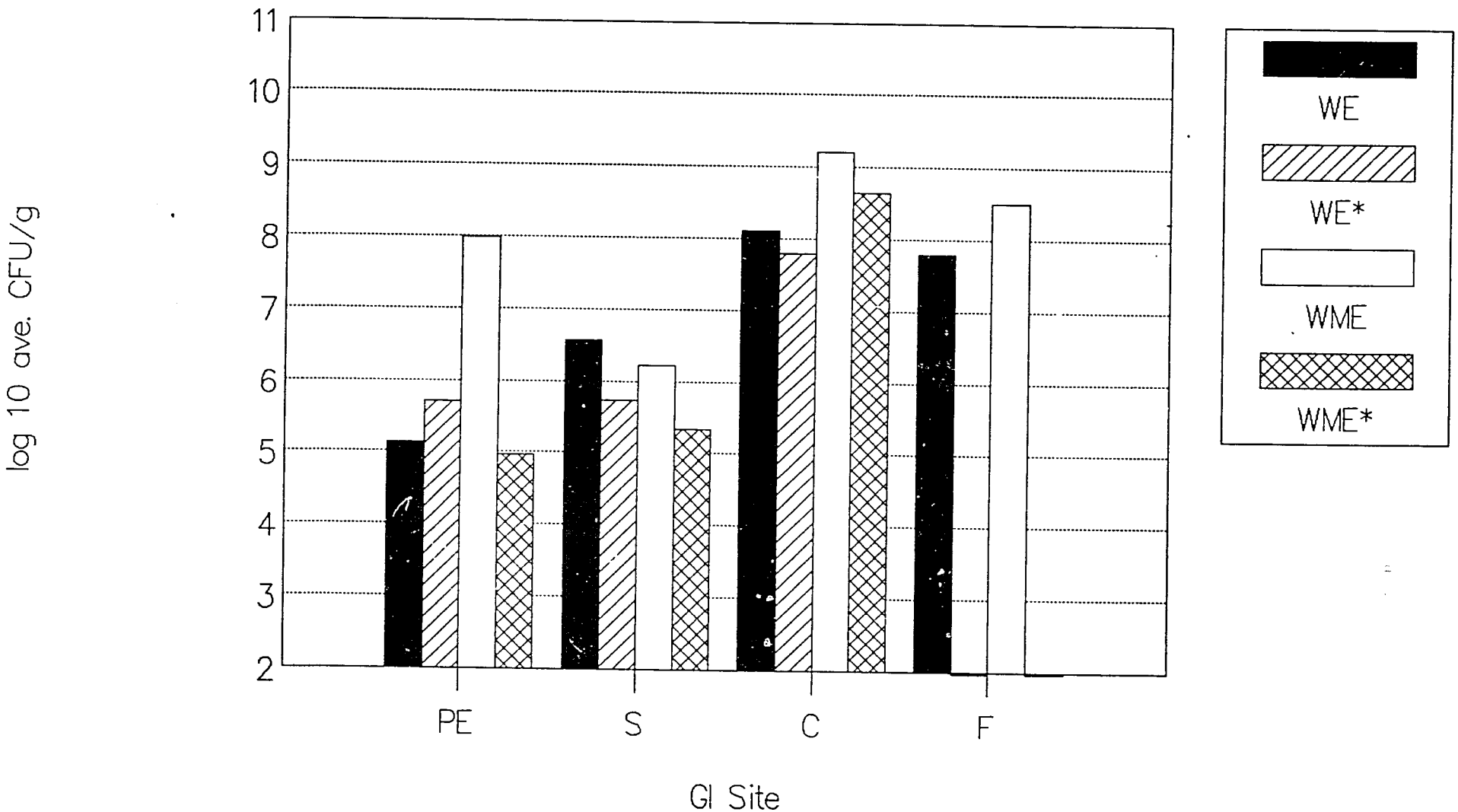
SF 2

Presence of E. coli in Experimental Groups



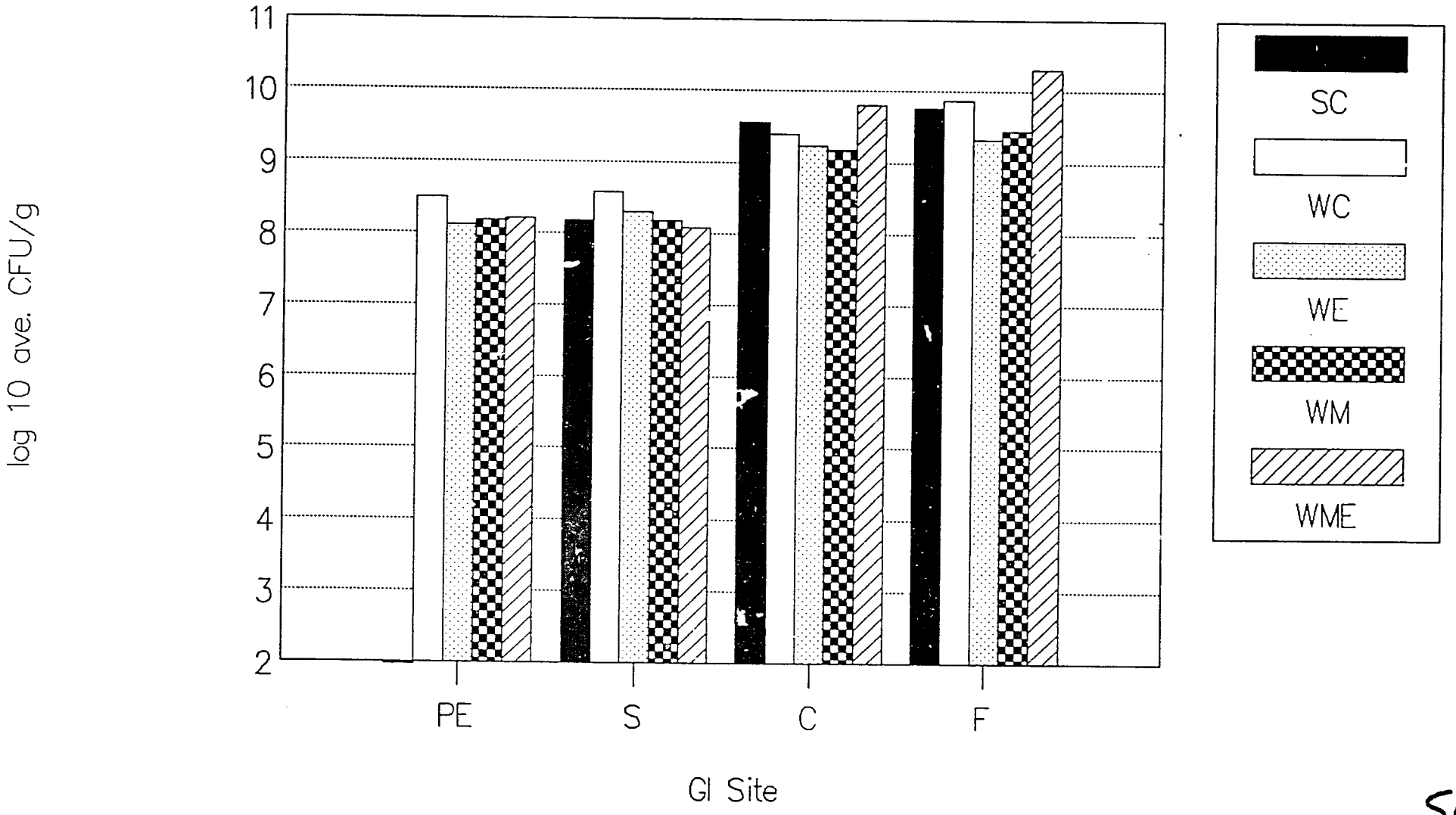
SF 3

Presence of E. coli in Experimental Groups



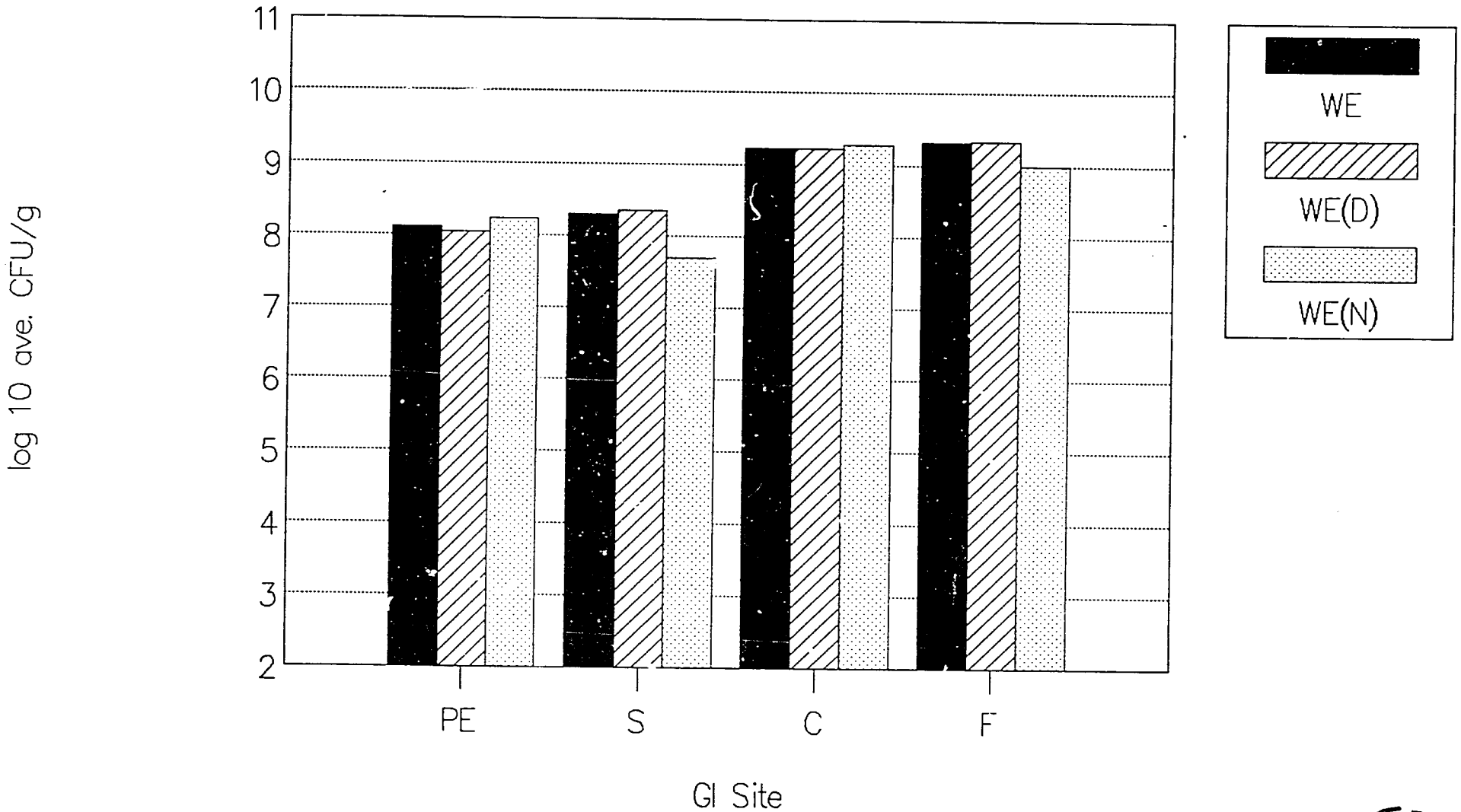
SF4

Presence of Lactic Acid Bacteria in Experimental Groups



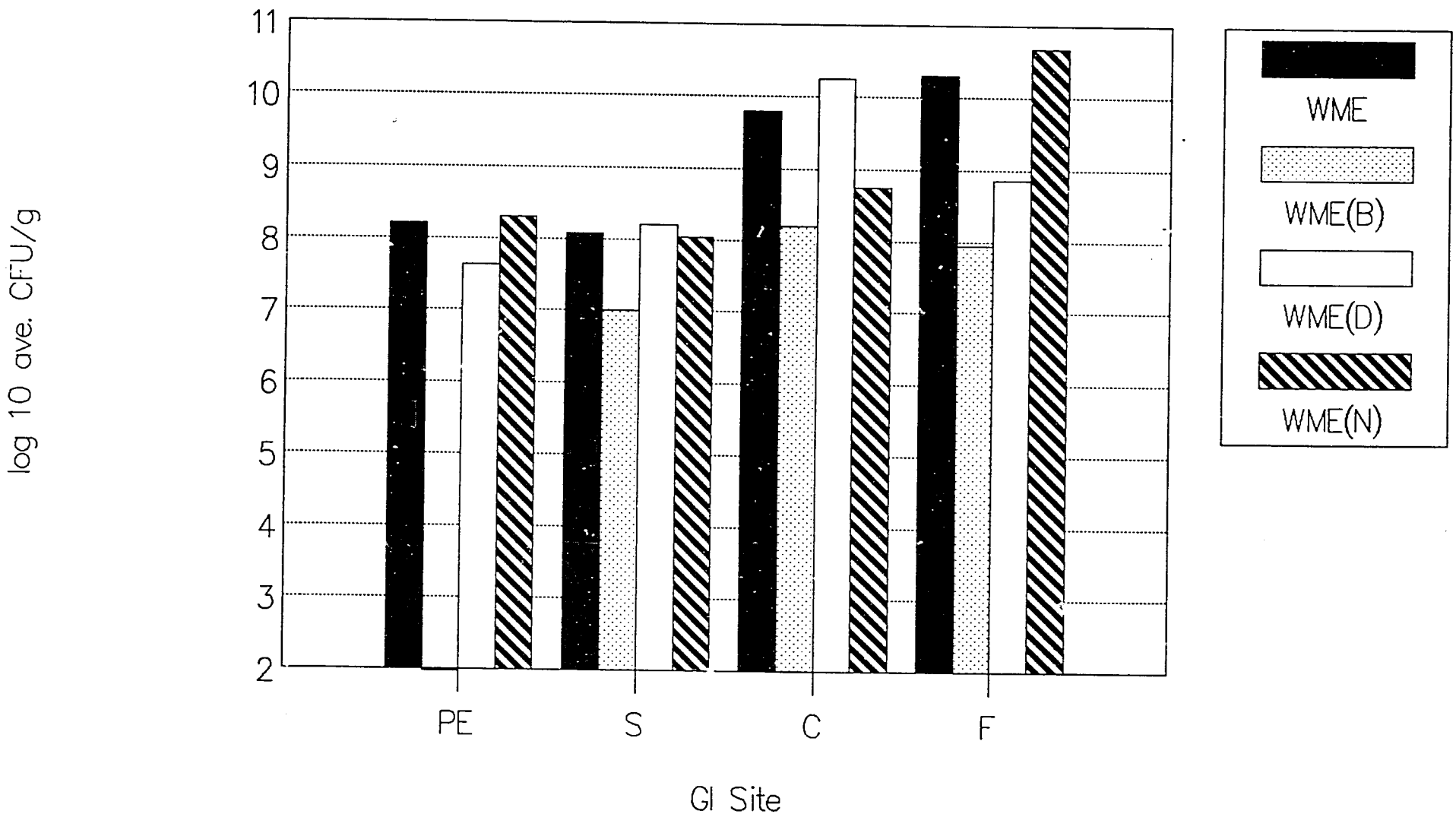
SF 5

Presence of Lactic Acid Bacteria in Experimental Groups



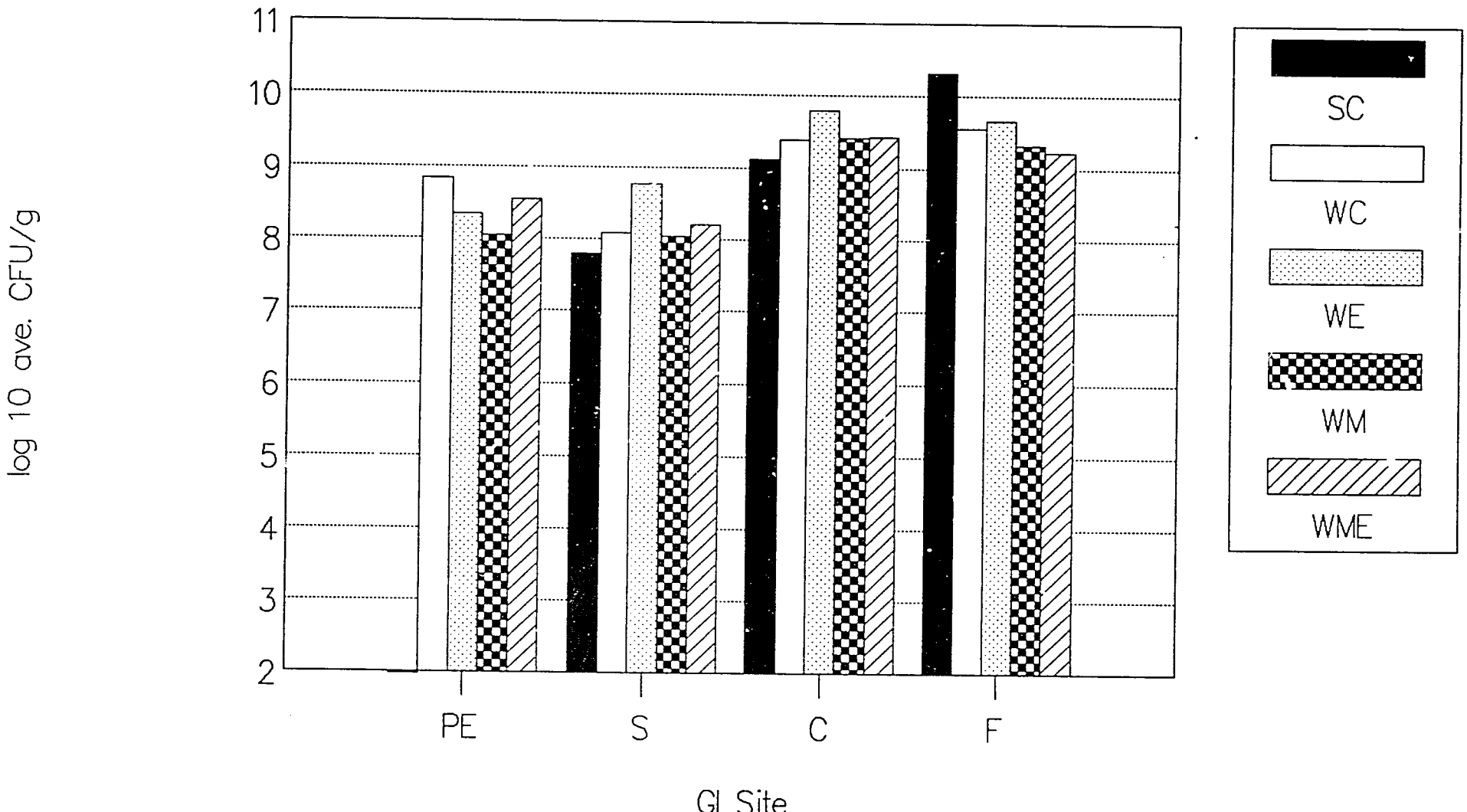
SF6

Presence of Lactic Acid Bacteria in Experimental Groups



SF7

Total Anaerobes Present in Experimental Groups



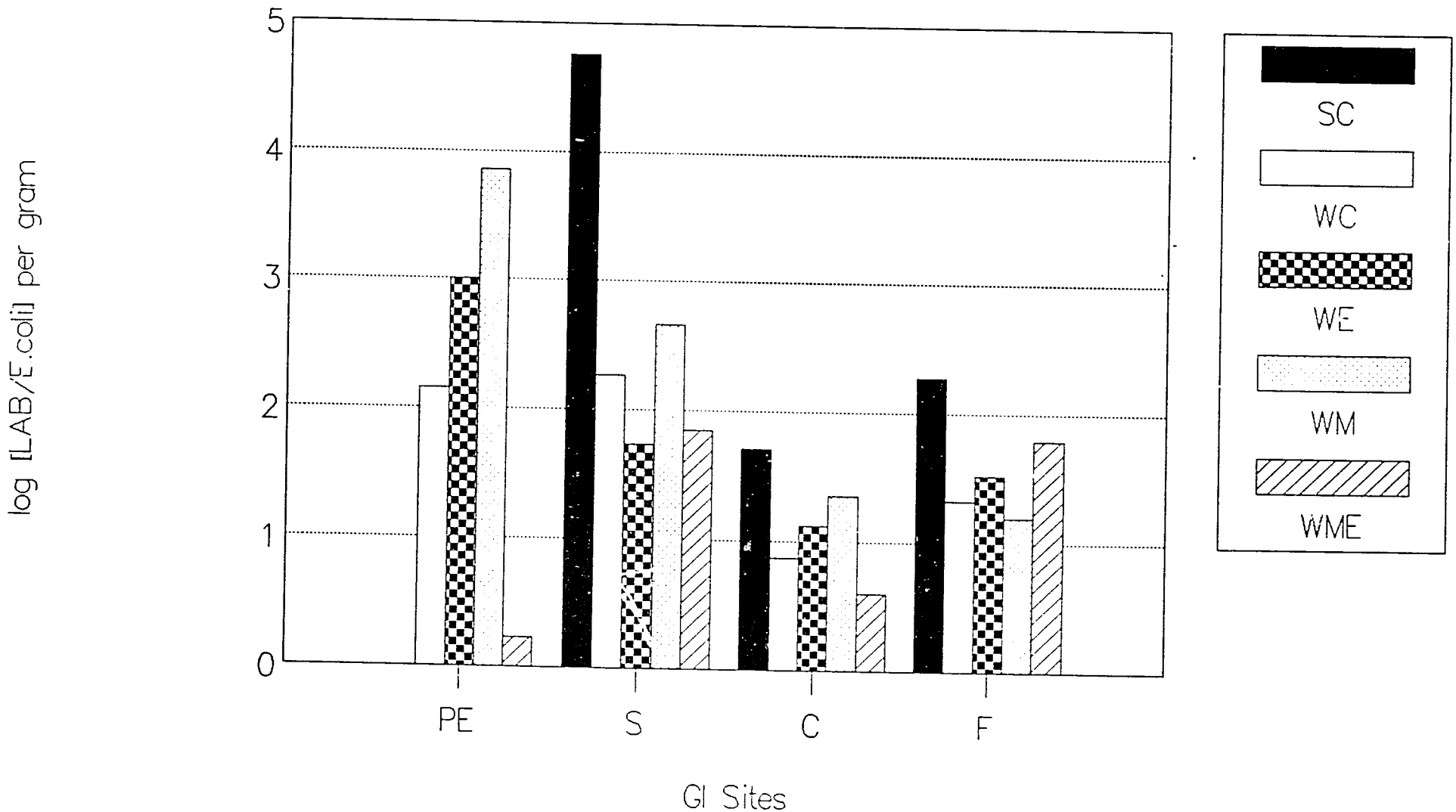
SF 8

SUMMARY FROM MEDIPHARM TRIALS 1, 2, AND 5
Sept. 28, 1989

RATIO OF LACTIC ACID BACTERIA TO E. COLI
IN EXPERIMENTAL GROUPS

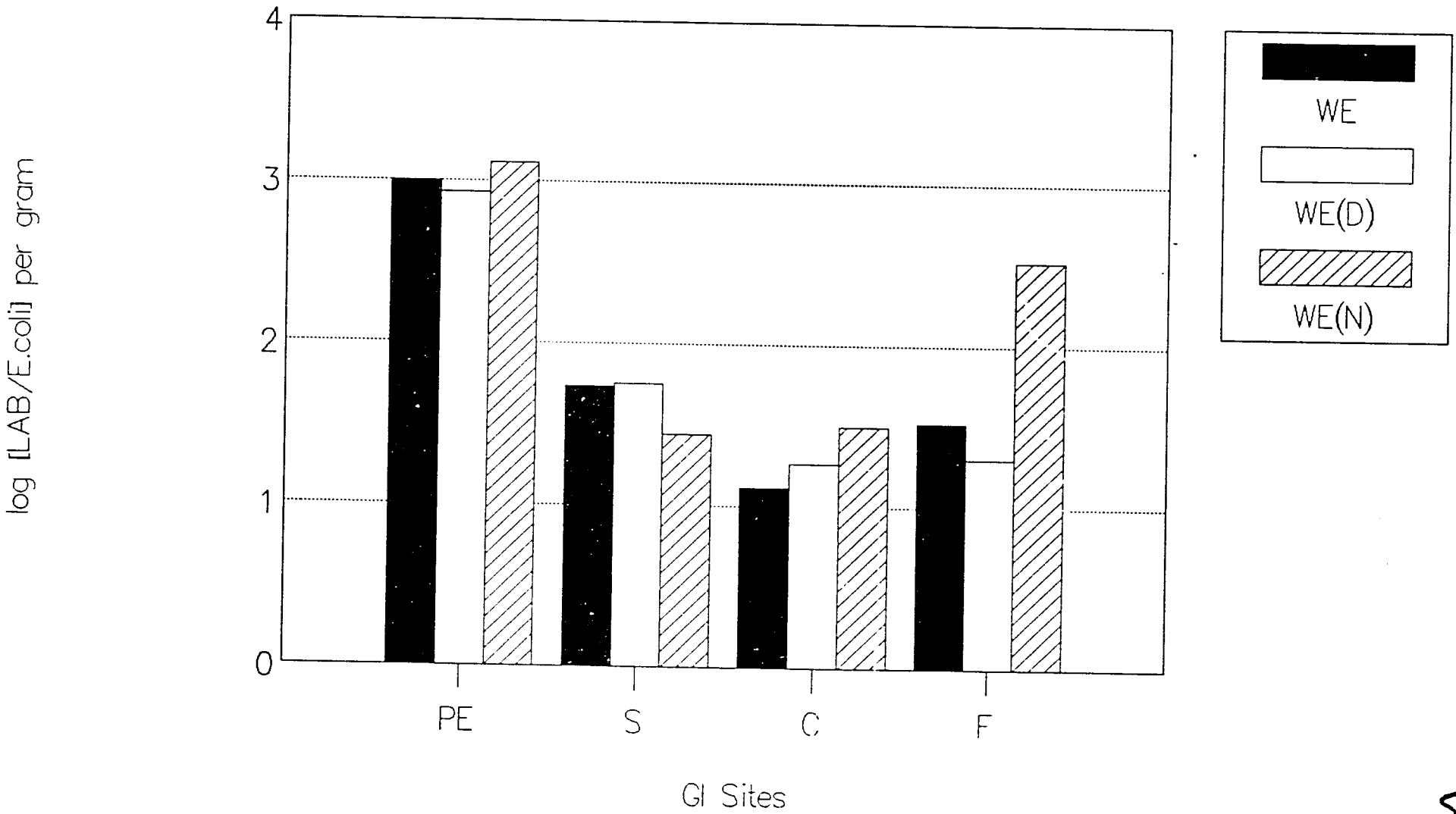
GROUP	log (LAB/E. coli) per gram PE	S	C	F
SC	ERR	4.8	1.7	2.3
WC	2.1	2.3	0.9	1.3
WE	3.0	1.7	1.1	1.5
WE(D)	2.9	1.7	1.3	1.3
WE(N)	3.1	1.4	1.5	2.5
WM	3.9	2.7	1.4	1.2
WME	0.2	1.8	0.6	1.8
WME(B)	ERR	1.5	-0.0	-0.1
WME(D)	2.8	2.6	0.7	-0.1
WME(N)	0.2	1.6	0.2	1.8
WC*	4.7	2.2	1.6	ERR

Ratio of LAB to E. coli in Experimental Groups



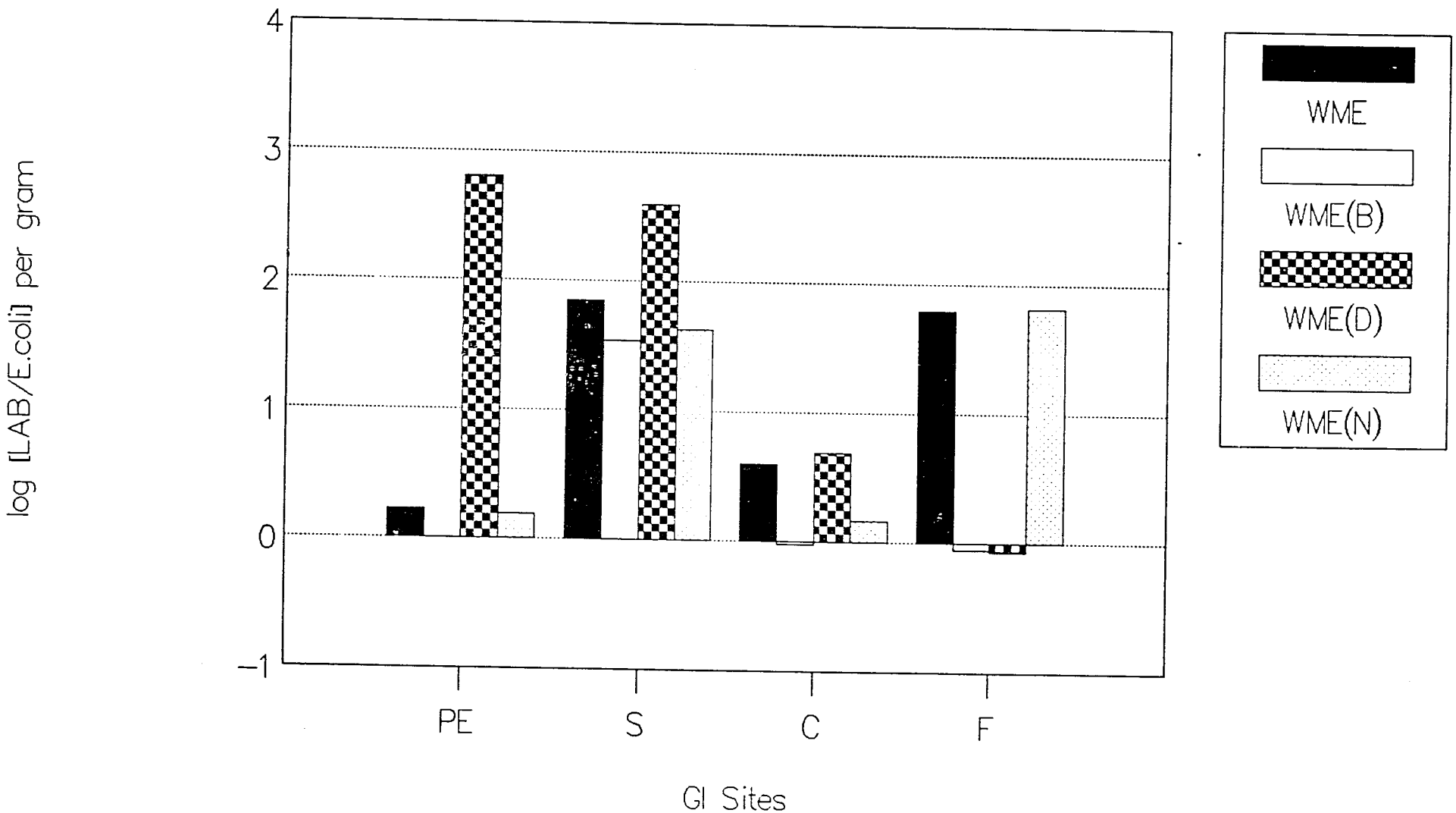
SFA

Ratio of LAB to E. coli in Experimental Groups



SFB

Ratio of LAB to E. coli in Experimental Groups



SFC

Sumary of Observations of Clinical Manifestations of Diarrhea in Control piglets (WC) used in studies with lab strain (2CA) (Trials 4 and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL
42-5	-	-	-	-	-	-		
69-1	-	-	-	-*				
70-11	-	-	-	-	-	-		
71-8	-	-	-	-	*			
71-9	+/- (3.0)	-	-	-	-*			
72-3	-	-	-	-	-	-	-	
74-7	-	-	-	-	-*			
76-5	-	-	-	-	-	-*		
77-6	-	-	-	-*				
78-6	-	-	-	-	-	-	-	
79-3	-	-	-	-	-	-	-	
	0/11 (1+/-)	0/11	0/11	0/11	0/9	0/6	0/3	0/11 (1+/-)

Sumary of Observations of Clinical Manifestations of Diarrhea in piglets challenged with E. coli (WE) during studies with lab strain (2CA) (Trials 4 and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed]

PIG#	-2	-1	0	+1	+2	+3	+4	TOTAL
71-7	-	-	-	-	-	-		
18-11	-	-	-	+/- (3.0)	-*			
18-6	-	-	-	-*				
71-3	-	-	-	-	-	-		
18-5	-	-	-	+(2.0) *				
69-4	-	-	-	+(1.0)	-	-		
71-6	-	-	-	+(2.0)	+(1.5)	+(2.0)		
69-9	-	-	-	-	-*			
71-3	-	-	-	+(2.5) *				
71-8	-	-	-	+(2.0)	+(2.0)	+(2.0)	-	
77-4	-	-	-	-	-	+(1.5)		
78-4	-	-	-	+(2.0) *				
75-2	-	-	-	+(1.0)	-	-	-	
77-3	-	-	-	-	-	-*		
75-7	-	-	-	+(2.5)	+(1.5) *			
	0/15	0/15	0/15	8/15 (1+/-)	3/11	3/8	0/3	9/15 (1+/-)

Sumary of Observations of Clinical Manifestations of Diarrhea in piglets receiving lab strain 2CA (WL) (Trials 4 and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed; ** = deleted from study]

PIG #	-2	-1	0	+1	+2	+3	+4	TOTAL
18-15	-	-	-	-	-*			
69-3	-	-	-	-	-	-		
18-3	-	-	-	+(1.5) *				
14-4	-	+(2.0) **						
71-5	-	-	-	-	-*			
18-7	-	-	-	-	-	-		
71-2	-	-	-	-*				
70-8	-	-	-	+(2.0)	-	-		
71-10	-	-	-	-	-	-	-	
73-2	-	-	-	-*				
74-12	-	-	-	-	-	-*		
75-5	-	-	-	-	-	-	-	
77-2	-	-	-	-	-*			
78-5	-	-	-	-	-	-*		
79-4	-	-	-	-	-	-	-	
	0/15	0/15	0/14	2/14	0/11	0/8	0/3	2/14

Summary of Observations of Clinical Manifestations of Diarrhea in piglets receiving lab strain 2CA followed by challenge with E. coli (WLE) (Trials 4 and 5). [4.0 (-), normal, firm stool; 3.0 (+/-), borderline; 2.0 (+), liquid stool; 1.0 (+), watery stool; * = sacrificed; ** = found diseased]

PIG #	-2	-1	0	+1	+2	+3	+4	TOTAL
70-9	-	-	-	-	+/- (3.0)	-		
69-8	-	-	-	-	-*			
14-10	-	-	-	-	-	-		
71-5	-	-	-	+/- (3.0)*				
70-3	-	-	-	+/- (3.0)	**			
71-9	-	-	-	+/- (3.0)	-	**		
69-7	-	-	-	-	+(2.0) *			
69-6	-	-	-	-*				
68-11	-	-	-	-	-	-		
18-13	-	-	-	+(2.0)	-	-		
18-16	-	-	-	-	-	-		
14-12	-	-	-	-	-	-		
18-8	-	-	-	-	-	-		
71-6	-	-	-	-	-	-*		
74-6	-	-	-	-	-	-	-	
77-5	-	-	-	-	-*			
78-2	-	-	-	-	+(2.5)	+(1.5)	-	
69-9	-	-	-	+(2.0) *				
PIG #	-2	-1	0	+1	+2	+3	+4	TOTAL

78-8	-	-	-	-	-	-	-	
71-5	-	-	-	-*				
	0/20	0/20	0/20	2/20 (3+/-)	2/16 (1+/-) (1**)	1/12 (1**)	0/3	4/20 (4+/-) (2**)

Distribution of *Escherichia coli* in the gastrointestinal tract of piglets during Strain 2CA Study (Trials 4 and 5).

GROUP	CFU/g PE	S	C
WC	2.1×10^6	3.3×10^4	8.9×10^7
WE	1.1×10^6	1.3×10^7	6.2×10^8
WE(B)	5.2×10^5	1.7×10^7	9.0×10^8
WE(D)	3.4×10^5	2.3×10^5	1.7×10^8
WE(N)	2.2×10^6	2.7×10^7	1.1×10^9
WL	9.7×10^4	9.7×10^4	5.1×10^7
WL(D)	1.4×10^5	1.8×10^5	3.0×10^6
WL(N)	9.1×10^4	8.5×10^4	5.8×10^7
WLE	7.6×10^6	3.5×10^6	2.6×10^8
WLE(B)	7.5×10^4	2.5×10^3	1.2×10^7
WLE(D)	1.1×10^6	1.3×10^6	3.2×10^8
WLE(N)	1.2×10^7	5.1×10^6	2.9×10^8
WC*	5.6×10^3	6.7×10^4	1.2×10^7
WE*	4.9×10^5	5.3×10^5	6.2×10^7
WE(D)*	4.9×10^5	5.3×10^5	6.2×10^7
WLE*	2.2×10^5	4.5×10^6	4.3×10^8
WLE(D)*	9.8×10^4	1.3×10^7	1.2×10^8
WLE(N)*	2.9×10^5	2.8×10^5	5.9×10^8

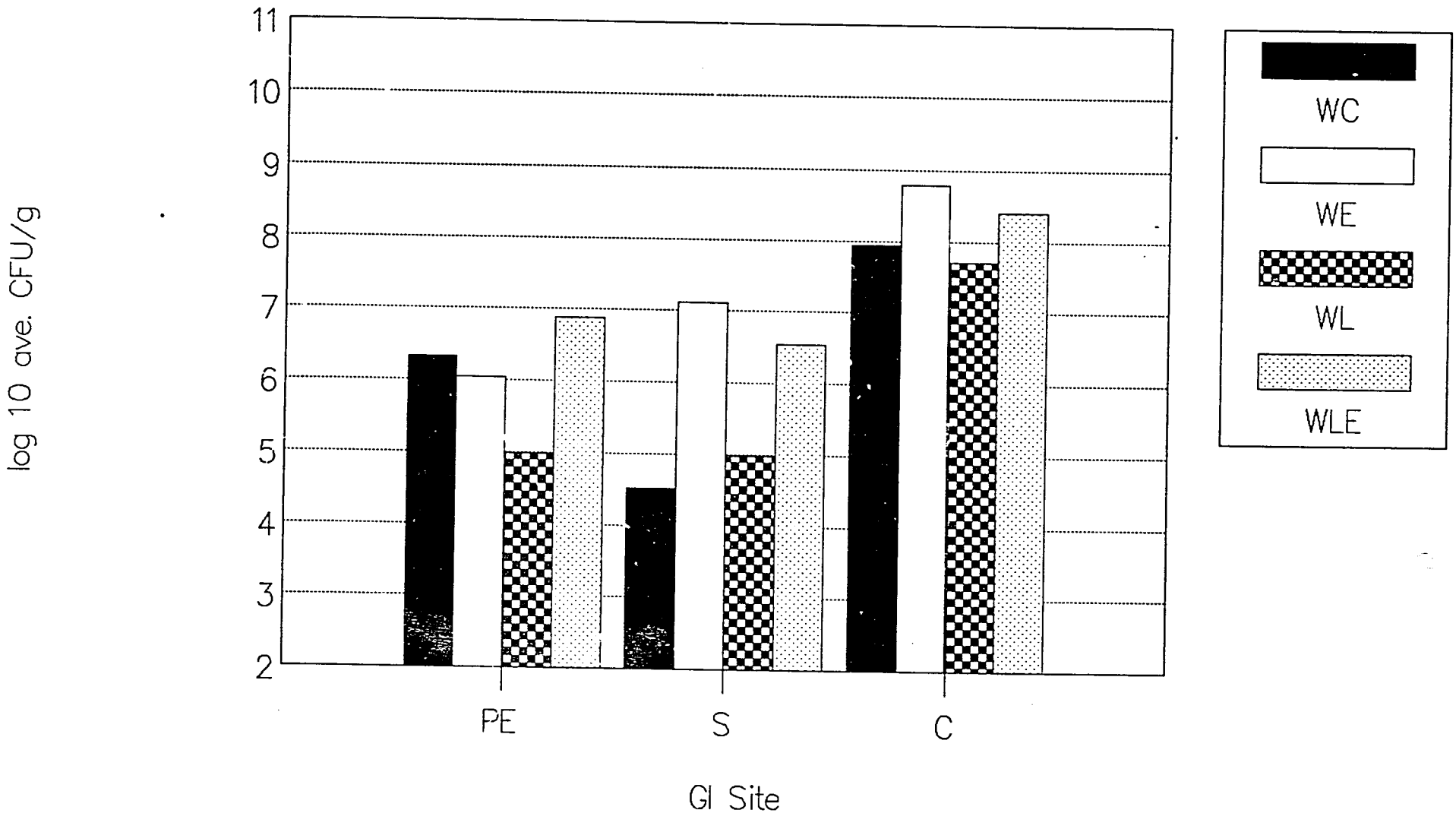
Distribution of lactic acid bacteria in the gastrointestinal tract of piglets during Strain 2CA Study (Trials 4 and 5).

GROUP	CFU/g PE	S	C
WC	2.5×10^8	7.2×10^7	1.0×10^9
WE	1.4×10^8	7.2×10^7	9.8×10^8
WE(B)	1.3×10^8	7.2×10^7	1.9×10^9
WE(D)	1.6×10^8	5.1×10^7	7.6×10^8
WE(N)	1.1×10^8	9.9×10^7	9.8×10^8
WL	2.5×10^8	1.4×10^8	2.2×10^9
WL(D)	1.6×10^8	1.8×10^8	2.9×10^9
WL(N)	2.6×10^8	1.3×10^8	2.1×10^9
WLE	1.8×10^8	6.7×10^7	5.5×10^8
WLE(B)	4.5×10^8	2.6×10^7	6.7×10^8
WLE(D)	2.1×10^8	3.5×10^7	1.3×10^9
WLE(N)	1.2×10^8	8.9×10^7	2.2×10^8
WC*	2.6×10^8	1.1×10^7	5.2×10^8
WLE*	3.3×10^8	1.1×10^6	1.1×10^9
WLE(D)*	1.5×10^8	2.4×10^6	1.2×10^9
WLE(N)*	4.2×10^8	4.8×10^5	1.1×10^9

Distribution of anaerobic bacteria in the gastrointestinal tract of piglets during Strain 2CA Study (Trials 4 and 5).

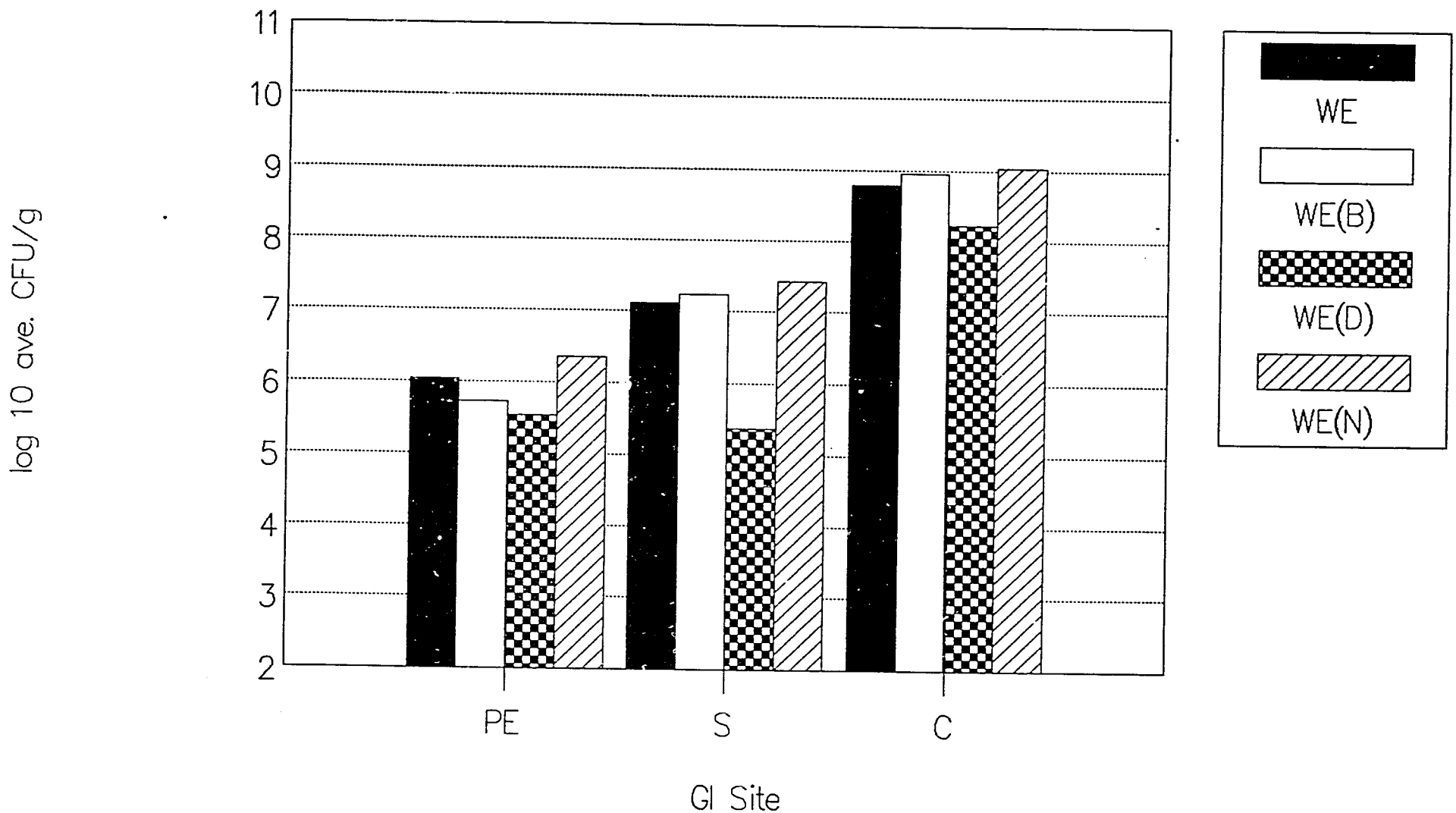
GROUP	CFU/g		
	PE	S	C
WC	5.0×10^8	8.2×10^7	2.0×10^9
WE	3.0×10^8	8.7×10^7	1.6×10^9
WE(B)	9.3×10^7	7.0×10^7	4.0×10^9
WE(D)	4.1×10^8	6.5×10^7	1.0×10^9
WE(N)	2.2×10^8	1.2×10^8	1.7×1[illegible]
WL	2.2×10^8	1.7×10^8	2.6×10^9
WL(D)	8.7×10^7	2.4×10^8	1.5×10^9
WL(N)	2.4×10^8	1.6×10^8	2.8×10^9
WLE	3.0×10^8	9.1×10^7	9.8×10^8
WLE(B)	6.0×10^8	2.8×10^7	3.3×10^8
WLE(D)	1.7×10^8	3.0×10^7	1.6×10^9
WLE(N)	2.9×10^8	1.3×10^8	6.0×10^8

Presence of E. coli in Experimental Groups

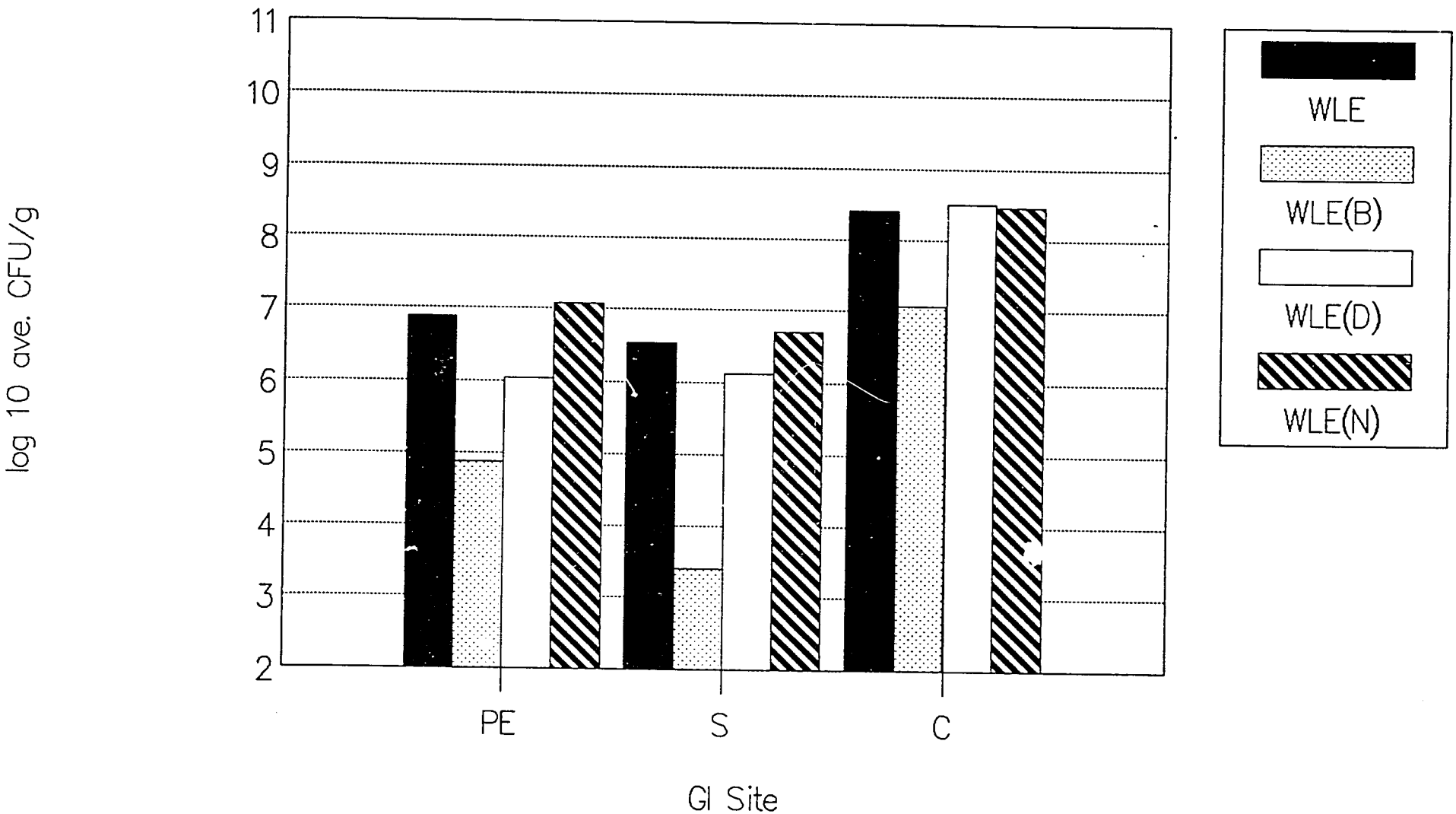


2CA 1

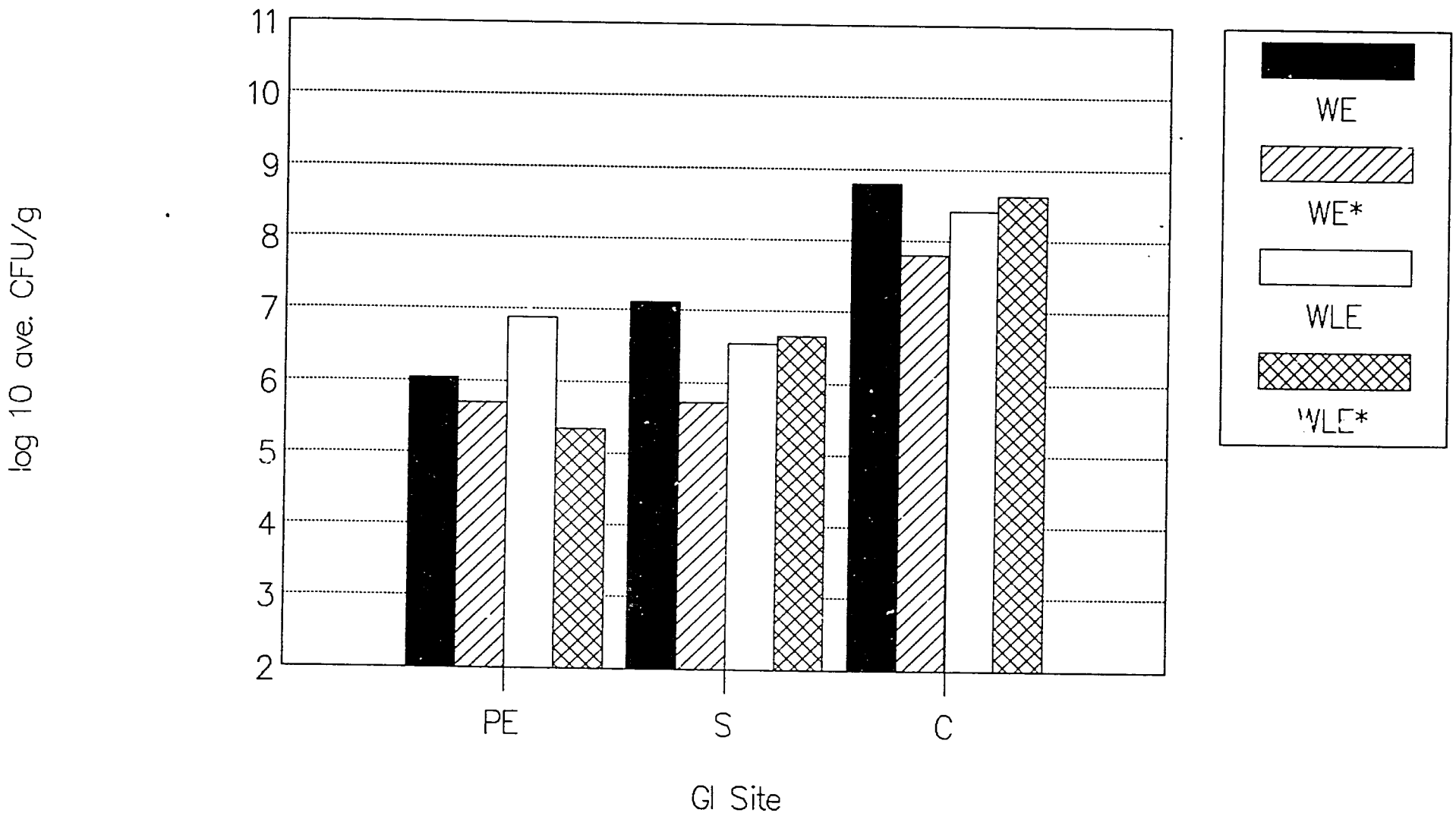
Presence of E. coli in Experimental Groups



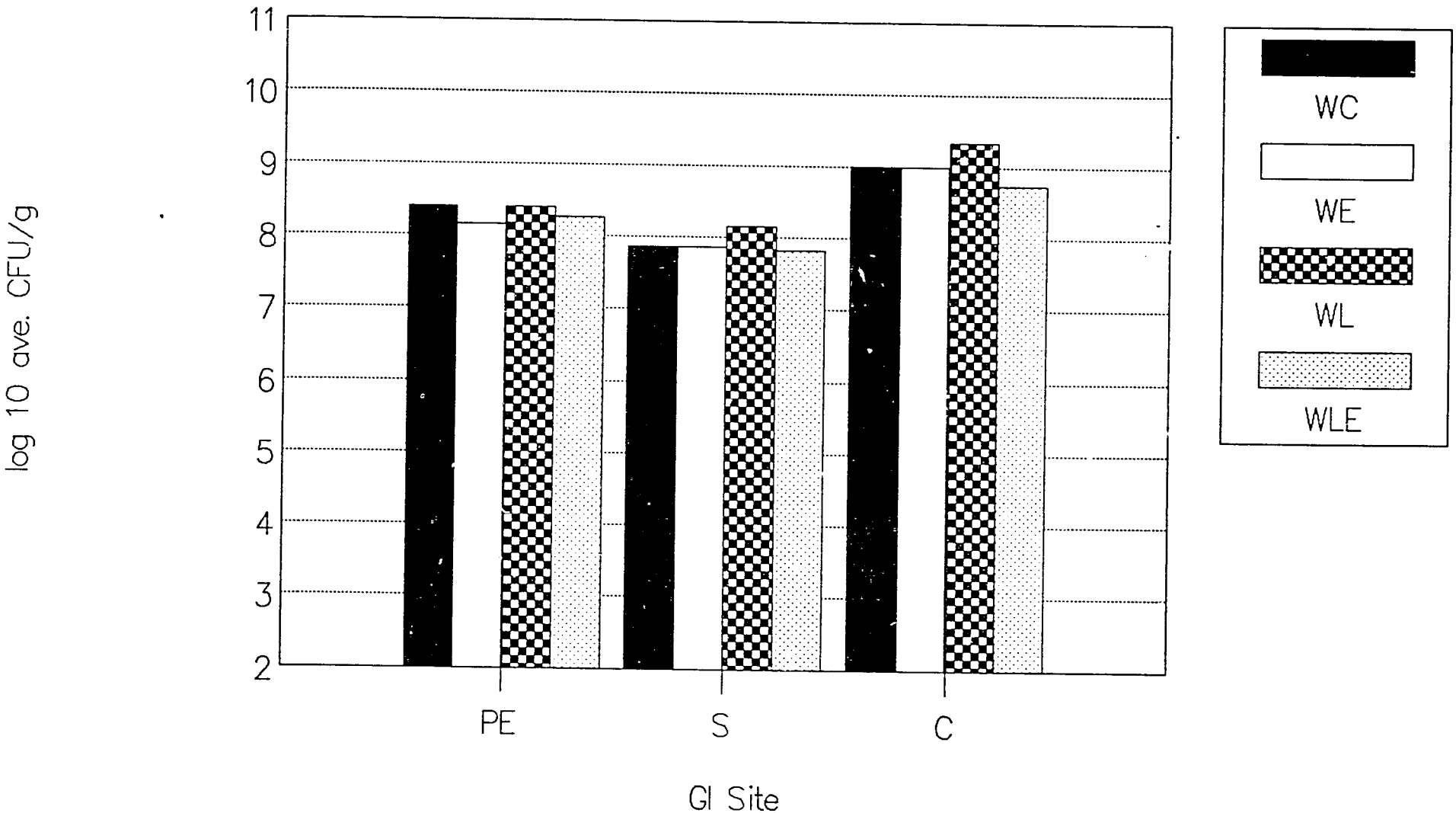
Presence of E. coli in Experimental Groups



Presence of E. coli in Experimental Groups

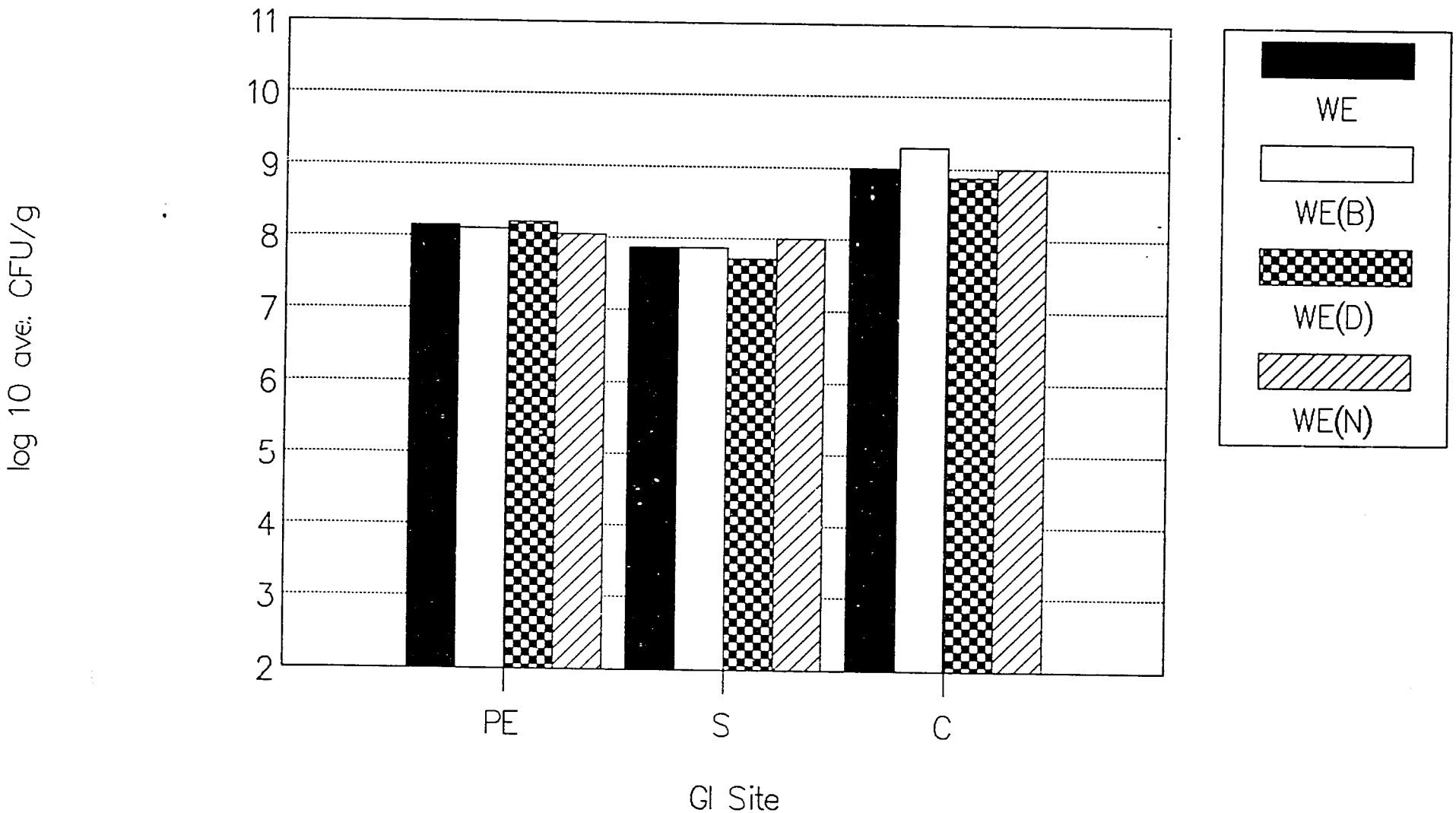


Presence of Lactic Acid Bacteria in Experimental Groups



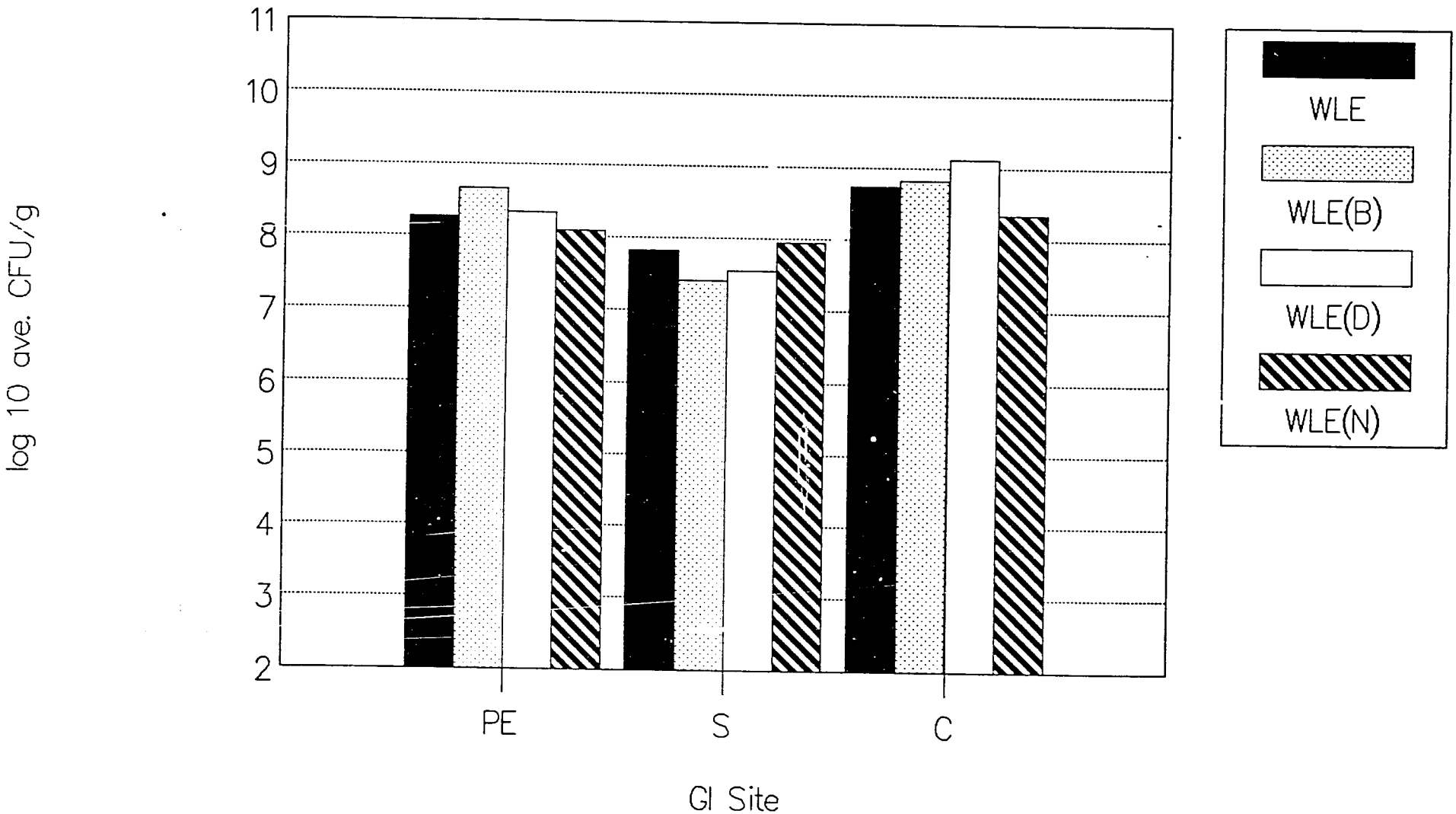
2CA 5

Presence of Lactic Acid Bacteria in Experimental Groups



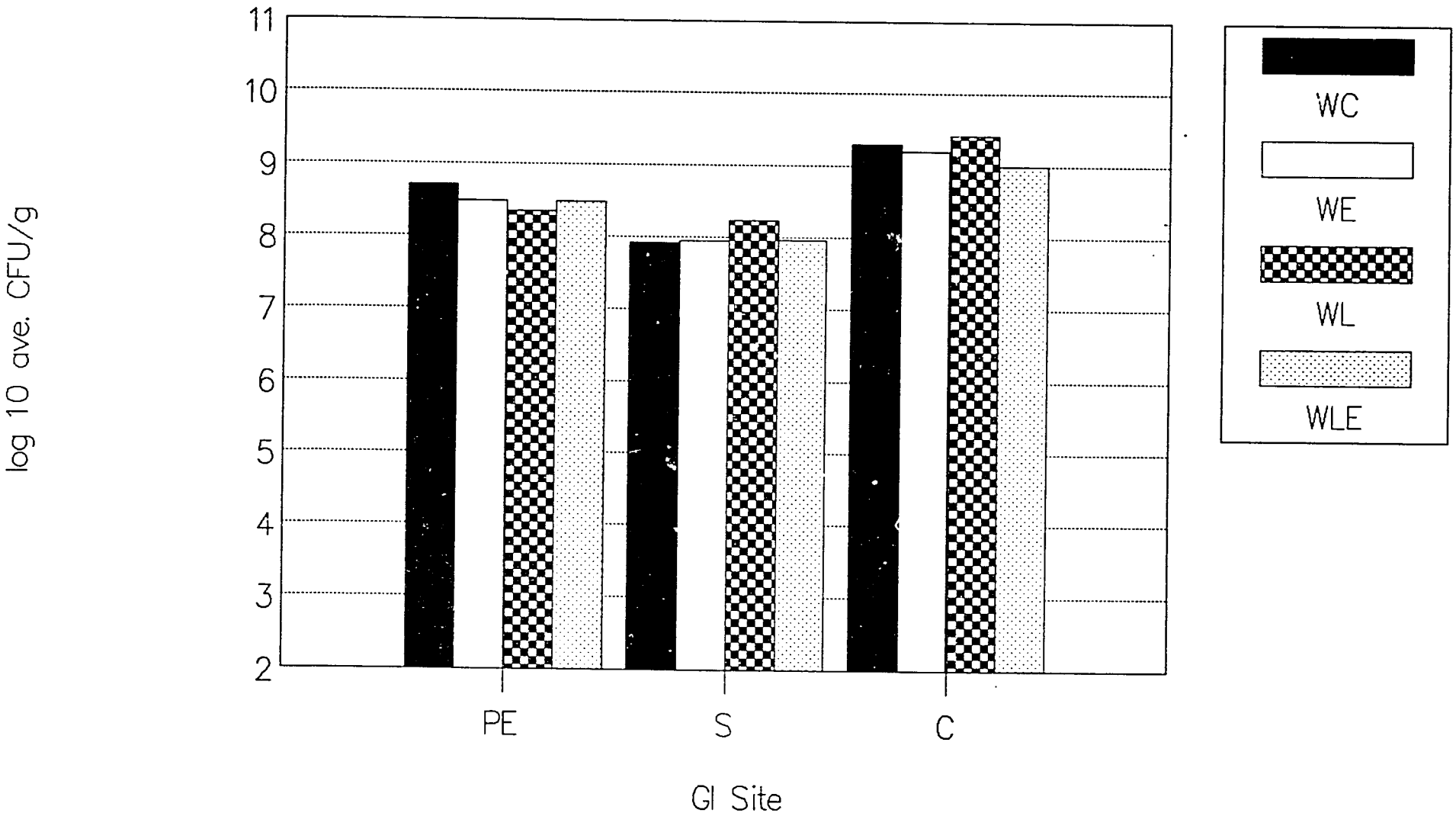
2CAL

Presence of Lactic Acid Bacteria in Experimental Groups



2CA 7

Total Anaerobes Present in Experimental Groups



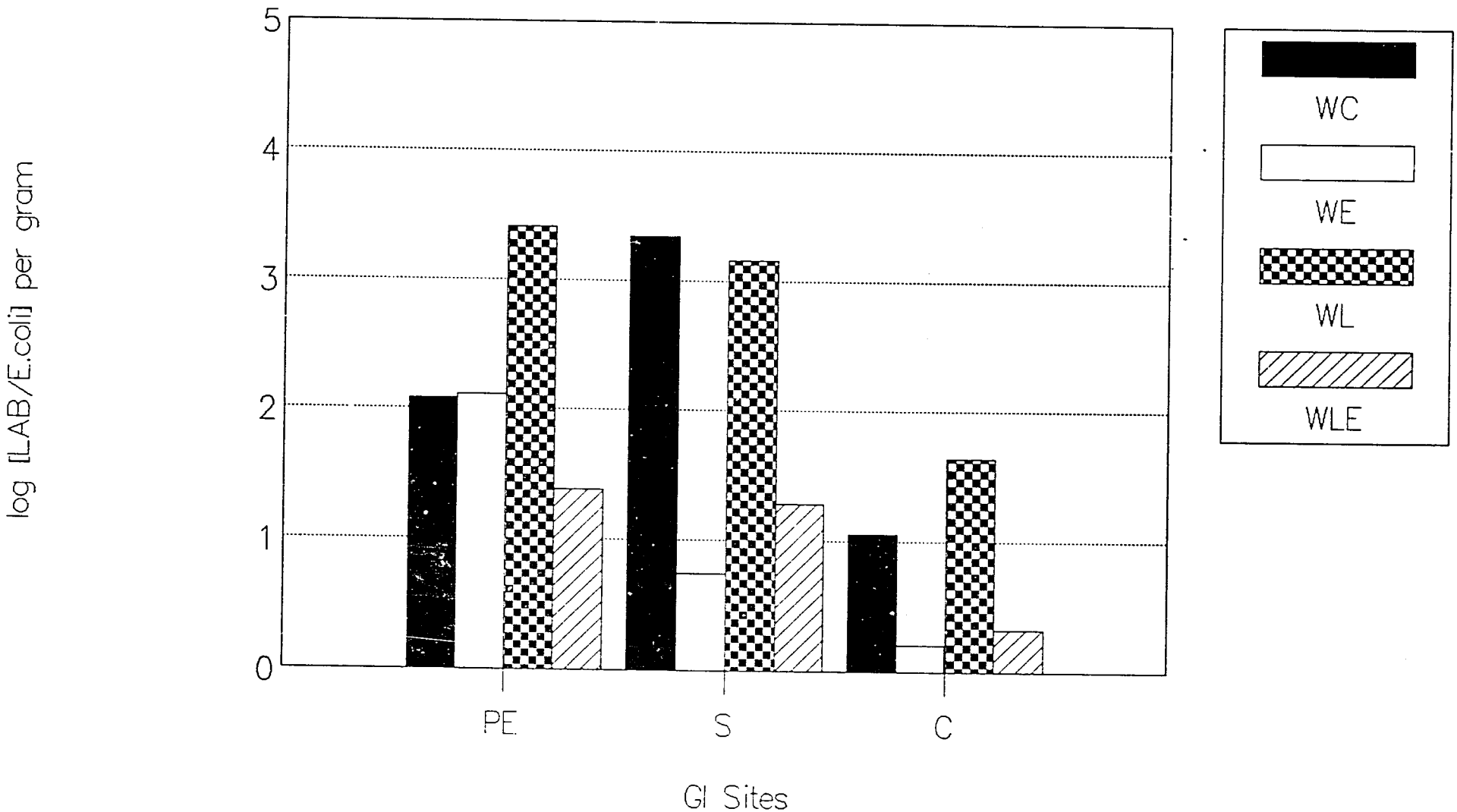
2CA 8

SUMMARY FROM LAB STRAIN 2GA TRIALS 4 AND 5
Sept. 28. 1989

RATIO OF LACTIC ACID BACTERIA TO E. COLI
IN EXPERIMENTAL GROUPS

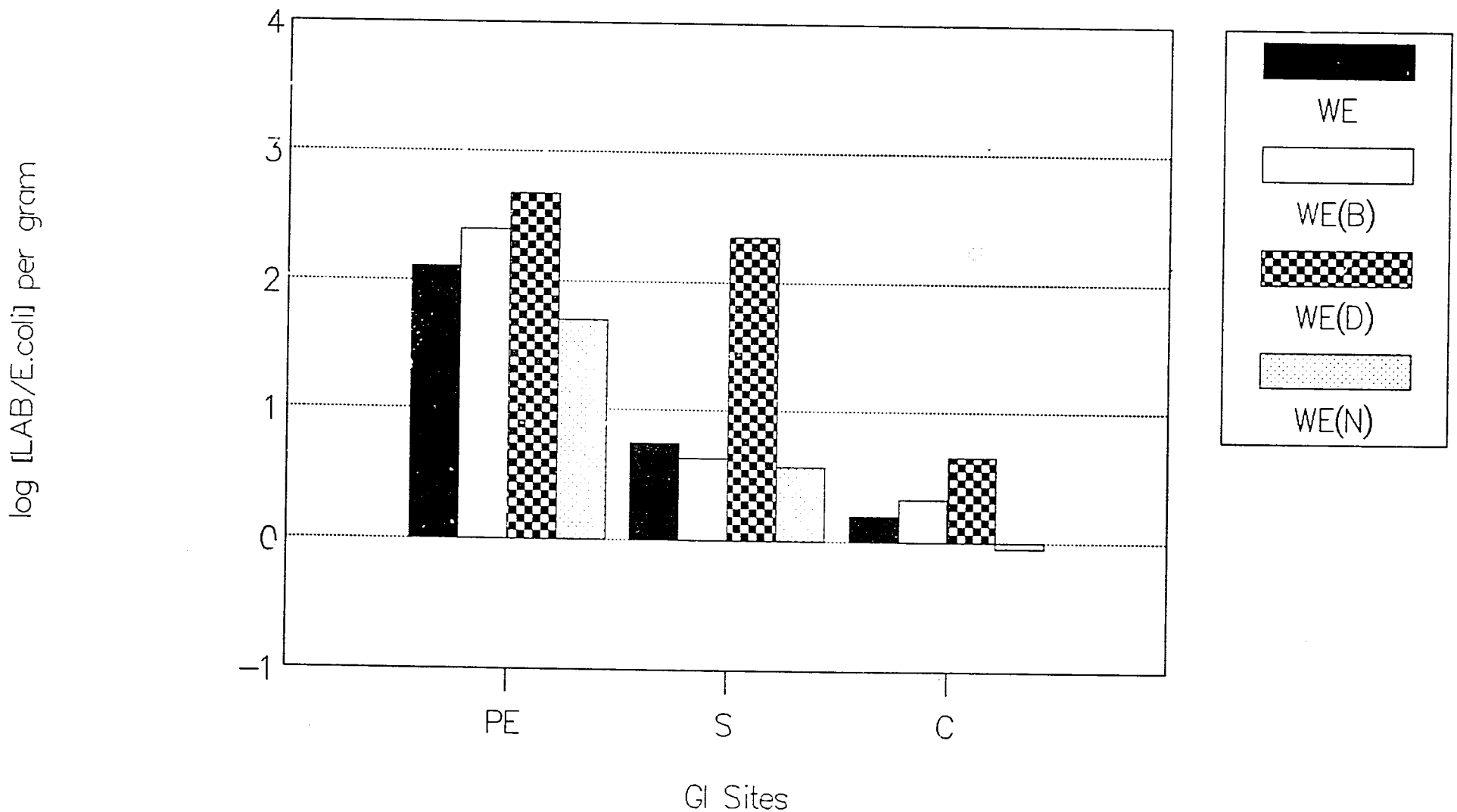
GROUP	log (LAB/E. coli) per gram PE	S	C
WC	2.1	3.3	1.1
WE	2.1	0.7	0.2
WE(B)	2.4	0.6	0.3
WE(D)	2.7	2.3	0.7
WE(N)	1.7	0.6	-0.1
WL	3.4	3.2	1.6
WL(D)	3.1	3.0	3.0
WL(N)	3.5	3.2	1.6
WLE	1.4	1.3	0.3
WLE(B)	3.8	4.0	1.7
WLE(D)	2.3	1.4	0.6
WLE(N)	1.0	1.2	-0.1
WC*	4.7	2.2	1.6
WLE*	3.2	-0.6	0.4
WLE(D)*	3.2	-0.7	1.0
WLE(N)*	3.2	0.2	0.3

Ratio of LAB to E. coli in Experimental Groups



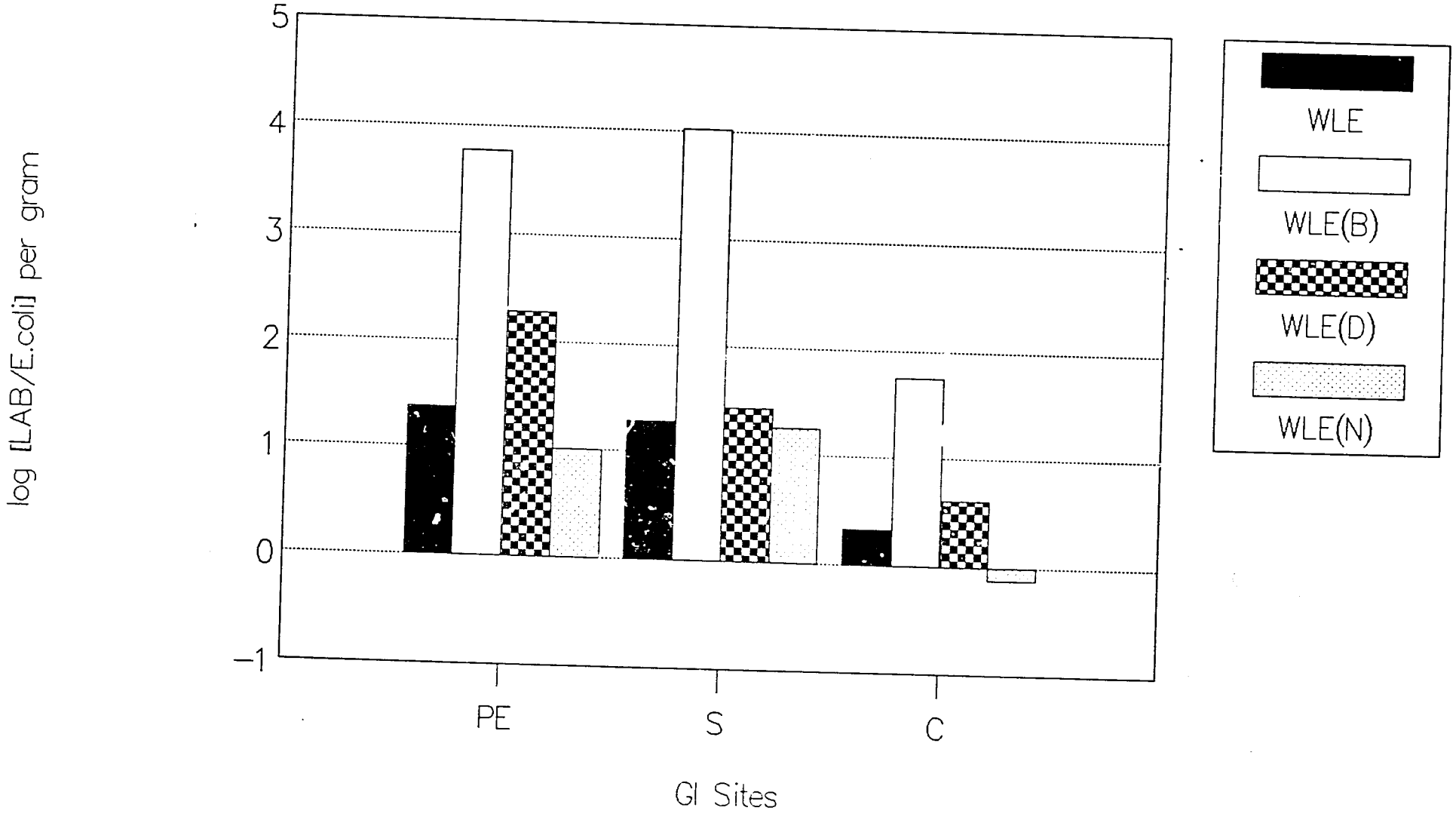
LCAA

Ratio of LAB to E. coli in Experimental Groups



LAB

Ratio of LAB to E. coli in Experimental Groups



2CAC