BIODIVERSITY OF INTESTINAL MICROFLORA IN SEVERELY MALNOURISHED CHILDREN WITH CHOLERA

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SUBMITTED BY:

SHIRAJUM MONIRA

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UNIVERSITY OF DHAKA

DHAKA-1000, BANGLADESH

CERTIFICATE

This is to certify that the thesis entitled "Biodiversity of intestinal microflora in severely malnourished children with cholera" was carried out by Shirajum Monira, Roll No. 013; Session: 2003-2004 for the fulfillment of the degree of Doctor of Philosophy (Science), from the Department of Microbiology, University of Dhaka, Bangladesh.

This work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of Ph.D degree.

Professor M Mozammel Hoq
Department of Microbiogy
University of Dhaka
Dhaka-1000, Bangladesh.

Dr. Motiur Rahman
Scientist & Head
RTI/STI Laboratory
Laboratory Sciences Div
ICDDR,B
Mohakhali, Dhaka.

Professor A.K.Azad Chowdhury
Department of Clinical Pharmacy
& Pharmacology
University of Dhaka
Dhaka-1000, Bangladesh.

Abstract

Children with cholera are often associated with dehydration and malnutrition. Recent nutritional interventions have targeted colonic functions in rehydrating patients with cholera and during recovery from malnutrition with the assumption that the effects will be influenced by metabolism of complex carbohydrates by colonic bacteria. In addition to stimulating small intestinal water absorption by glucose, complex carbohydrates have been proposed for additional rehydration and nutrition through short-chain fatty acids (SCFAs) produced in colon. However, study of colonic bacterial diversity, their number and metabolic activity during cholera is not known.

The aim of the study was to assess the diversity, concentration and proportion of faecal microbiota and concentration of SCFA during rehydration and nutritional therapy periods of severely malnourished children with cholera receiving oral rehydration solutions (ORS) containing one of the 3 substrates: rice, glucose or glucose with amylase-resistant starch (ARS).

Serial faecal samples were collected from 30 severely malnourished children with cholera until completion of rehydration and partial nutritional recovery. In addition 11 severely malnourished children without diarrhoea and 6 healthy children were enrolled in the study. PCR, using universal primers for 16S rDNA was performed on chromosomal DNA extracted from the stool samples, and the products were separated by TTGE. The number and proportion of bacteria in faeces were determined by FISH combined with flow cytometry. HPLC was used for identification and quantification of SCFAs after extraction by diethyl ether.

The *Vibrio cholerae* band was detected in all children at enrolment and the average number of bands was 7.7 ± 0.5 (mean ± sem). The *V. cholerae* band was disappeared within 2 days. On day 2, a rapid and significant increase in the band numbers was observed which was followed by a steady increase up to day 28. After full recovery from cholera and partial recovery from malnutrition, the number of bands (11.5 ± 0.6, at day 28) was lower than in healthy children (22.2 ± 0.4) but higher than severely malnourished children without cholera (8.1 ± 1.1). On day 3, the number of bands was greater with rice or amylase resistant starch than with glucose ($P<0.05$). Before treatment the population of bacteria and concentration of SCFAs was very low (7.9 ± 0.08 log10/g and 4.7 ± 0.6 mmol/kg, respectively). In the first 3 days of treatment, the stool output was more reduced and both

the concentration of bacteria and SCFA increased faster in the rice-ORS group than in the glucose-ORS group, and to a lesser extent in the glucose-ORS plus ARS group. A significant difference in SCFAs concentration was observed between rice and glucose-ORS group ($p = 0.047$) and between rice and ARS-ORS ($p= 0.039$) on day 3. There was a trend to have higher proportion of *Bacteroides sp.*, *Bifidobacterium sp.* and *F. prausnitzi* groups in rice ORS; *Bacteroides sp*, *C. coccoides*, and *Atopobium sp.* in glucose-ORS-ARS group; and *Lactobacillus-Enterococcus* group in glucose-ORS group on day 3. The duration to recovery of malnutrition was not different between the groups. At day 28, the bacterial population did not reach to healthy level. The SCFA concentration reached healthy level in the rice-ORS group while it remained at the malnutrition level with glucose and glucose-ORS plus ARS being intermediate.

The finding suggests that bacterial diversity was markedly reduced in severely malnourished children and had little effect during cholera. Clinical rehydration was associated with an increase in both bacterial population and diversity and SCFA concentrations, with all 3 carbohydrates in ORS. Better results were observed in the children receiving rice-ORS. Normal nutritional status was associated with further increase in bacteria and SCFAs.

The human intestinal tract harbours a complex microbial community which plays a major role in nutrition and health. The methods validated and used in this study will provide us a strong basis for further analysis of gut microbiota in different aspects e.g., intervention of new prebiotic or probiotic substances or nutritional problem in different disease conditions in children and adult in Bangladesh.

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Abbreviations

µl	: microlitre
cfu	: colony forming unit
et. al.	: and others
g	: gram
°C	: degree centigrade
rr	: ramp rate
SCFA	: Short Chain Fatty Acid
FISH	: Fluorescent In situ Hybridization
HPLC	: High Performance Liquid Chromatography
TTGE	: Temporal Temperature Gradient Gel Electrophoresis
ARS	: Amylase Resistant Starch
ORS	: Oral Rehydration Solution
LPS	: Lipo Polysaccharide
GI Tract	: Gastro Intestinal Tract
NSP	: Non starch polysaccharide
CFTR	: Cystic Fibrosis Transmembrane regulator
SGLT	: Na^+, glucose co transporter
sem	: standard error of mean
WHO	: World Health Organization
ATCC	: American Type Culture Collection
BLAST	: Basic Local Alignment Search Tool
rDNA	: ribosomal deoxy-ribonucleic acid
rRNA	: ribosomal ribo-nucleic acid
PBS	: Phosphate Buffered Saline
EDTA	: Ethylene Di-amine Tetra Acetic acid

Chapter I

Introduction

1.0 Introduction

The prognosis of severe malnutrition has been substantially improved with the use of nutritional therapy recommended by WHO. However, with an additional intestinal infection, including cholera, severe malnutrition can be still life threatening. In fact recommendation for severely malnourished children with cholera is not optimal (153). Cholera is one of the most common forms of diarrheal diseases and has long been known as a condition affecting many children in developing countries. However accurate health statistics remain scarce and it was not until the 1960s that diarrheal diseases were identified as a major cause of morbidity and mortality in children under five years of age. WHO estimates that malnutrition was associated with over half of all child deaths in developing countries in 1995. Among these deaths, diarrheal diseases account for 19% of cases (Focus on Nutrition, The State of the Worlds Children, 1998. http://www.unicef.org/sowc98/silent.htm.). Malnutrition and diarrhea are interlinked with each other and influences the morbidity progression of each other: malnutrition increases the severity and duration of diarrhea, and diarrhea may cause malnutrition (37). Diarrhea caused by *V. cholerae* can be adequately treated with oral rehydration salts (the WHO/UNICEF standard sachet) solution which is effective in correcting the dehydration and reducing the mortality. The basis for using standard glucose-ORS is that even during cholera glucose stimulates sodium and water absorption in the small intestine (36).

Since diarrhea is associated with further loss of weight and deficiency of macro- and micronutrients, early recovery as well as lessening severity from diarrhea will help to prevent further weight loss and deficiency of these nutrients in severely malnourished children. In addition, these children also have the depleted potassium stores and diarrhea further depletes the potassium concentration (221). Present WHO-ORS, with 90 mmolar sodium, is effective and safe in correcting dehydration in healthy and severely malnourished children (61, 137). Several attempts have been made to improve the quality of the ORS. This includes addition of potassium and other minerals to replace the deficiency associated with malnutrition as well as aggravated by diarrhea. This lead to the concept of Rehydration solution for Malnutrition (ReSoMal), and is being recommended by WHO (1).

The colon absorbs sodium against steep electrochemical gradients and can absorb up to 5 L of fluid daily (24). In diarrheal disease, the absorptive capacity of the colon compensates, for the secretion of fluid from the small intestine (142). In cholera, the secretion rate in small intestine is high and in addition there is decreased absorption of fluid in the large intestine (192). The decreased absorption phenomena can be reversed by short chain fatty acids in colon (156). Recent studies showed that if amylase resistant starch (ARS) is added to standard WHO-ORS, it can reduce the duration and severity of diarrhea in adults with cholera (158). Bacterial fermentation of unabsorbed carbohydrates in colon produces Short Chain Fatty Acids (SCFAs) (43). Starch that is resistant to digestion by amylases in the small intestine is found in small quantities in many cereals and is a good substrate for colonic fermentation process. The short chain fatty acids thus produced stimulate sodium and water absorption in the colon, which mimics glucose like activity in the small intestine (21). So, early recovery from diarrhea is expected to occur and this improves the appetite and food intake of the patient and subsequently causes faster growth and weight gain during the rehabilitation phase. In addition, a better recovery from malnutrition should be achieved through the energy provided by the SCFAs to the colon which is an additional benefit to the malnourished children. So, the colonic microflora is responsible for the metabolism of unabsorbed carbohydrates in the colon and thus offers a unique role in early recovery from diarrhea.

The human gastrointestinal tract constitutes an enormously complex ecosystem that includes both aerobic and anaerobic microorganisms and this large population suggests containing 30 to 40 dominant and 300 to 400 subdominant species of bacteria. These diverse groups of bacteria heavily populates (10^{10} –10^{12} ,cfu/g of luminal contents) the human intestine (133, 197, 217). However, these organisms are not randomly distributed throughout the intestine, but rather reside in specific ecologic niches. The flora of the stomach and proximal small intestine differ significantly from the terminal ileum and colon microflora. In colonic habitat the anaerobic bacteria outnumber the aerobes by a factor of at least 3 to 5 log 10. The obligate and facultative anaerobic bacteria are of diverse genera and range over a wide spectrum of taxonomic types (105). The consequence of colonic bacterial diversity during and after cholera in severely malnourished children is poorly known, so there is a knowledge gap on this issue. The present study is designed to test the hypothesis that amylase-resistant starch given during rehydration stimulates bacterial community in the colon, reduces the severity of cholera by stimulating the water and electrolyte absorption through the synthesis

of Short Chain Fatty Acids (SCFAs) and its colonic fermentation may be an additional source of energy during catch-up growth period of severely malnourished children with cholera. Thus the aim of the study is to measure the quantity and biodiversity of colonic flora as a function of time in children treated with one of the three ORSs i.e., i) Glucose-ORS, ii) Glucose-ORS containing amylase resistant starch and iii) cooked rice ORS. The diversity pattern obtained from cholera children will be compared both with malnourished children without cholera and healthy children.

Chapter II

Review of Literature

2. Review of Literature

2.1 Interaction between Diarrhea and malnutrition

Diarrheal diseases are a major cause of morbidity and mortality in infants and young children in the developing world. Although the use of glucose-electrolyte oral rehydration therapy (ORT) has dramatically reduced the mortality from dehydration caused by diarrhea, rates of morbidity as a result of this syndrome remain as high as ever (107). An estimated global mortality rate of diarrhea declined from approximately 4.6 million annual deaths during the mid-1980s to the current estimate of 1.87 million (range 1.6–2.1 million) in 2000 (29). Most of these deaths occur in children under the age of 5 years and in developing countries. In the same population segment malnutrition is a considerable health problem with prevalence rates estimated to range from 4% to 46% including 1% to 10% severely malnourished.

Diarrhea rarely operates alone. A bidirectional causal relationship is hypothesized wherein malnutrition predisposes the host to diarrhea, and conversely, diarrhea may exert a negative impact on nutritional status of individuals (37). Poor nutrition is often associated with more serious, and prolonged diarrhea and may lead to 20 - 40 g weight deficit/day (151). Actually the single effect of malnutrition or any infection is enormous and their combined effects are far greater than their individual contribution. Malnutrition magnifies the effect of disease through increasing the incidence and severity of the disease as well as by escalating the complication and duration of the disease. Globally, poorly nourished children suffer up to 160 days per year from illness. The risks of death in mildly, moderately and severely malnourished children are 2, 3 and 11 times higher respectively than the well nourished children (79).

During diarrhea, the body losses water and electrolytes in the form of liquid stool. Fluids can also be lost through vomiting, sweating, urination and perspiration/breathing. Dehydration occurs when these losses are not adequately replaced. Children are more likely die from diarrhea because they become dehydrated more quickly compared to adults. Malnutrition increases the risk of mortality because it is associated with disrupted water and electrolytes homeostasis. Diarrhea in malnourished children specially with protein calorie malnutrition is in great measure due to mucosal malnutrition (164). The distal intestinal mucosa requires

luminal substrates (dietary carbohydrates, proteins etc.) for epithelial metabolism/regeneration and where there is a paucity of these substrates, the intestinal cell growth is suppressed and the absorptive capacity of the colonic mucosa is diminished. Malnutrition also augment the recurrent episodes of diarrheal diseases, by exerting a negative impact on the barrier protection afforded by the skin and mucous membranes and by adverse alterations in specific and non-specific defense mechanism of host immune system (106).

Each episodes of diarrheal disease also has some nutritional impact on the children. Infection adversely affect nutritional status through reductions in dietary intake, increased catabolism and sequestration of nutrients that are required for tissue synthesis and growth (106). The reason for reduction of food intake during diarrhea may be due to child anorexia or maternal food-withholding behaviour or both (37). Child anorexia could be a consequence of clinical disturbances, including dehydration, electrolyte imbalance, fever, vomiting, or abdominal discomfort. Food-withholding behaviour by mothers could be a response to child anorexia or culturally ingrained changes of dietary practices either in response to illness or as an active component of disease management. In many situations, the net outcomes are the cessation of breast-feeding, compromises in the quality and quantity of weaning foods, and in some cases, reduction of food intake by lactating mothers themselves. Many studies have documented the malabsorption of macro- and micronutrients during acute diarrhea (37). Malabsorption of sugars (glucose, xylose, lactose) and fats is well substantiated and solid evidence exists for malabsorption of nitrogen, amino acids, and protein. Certain water and fat soluble vitamins (A, B_{12}, folate) and trace minerals (magnesium, zinc) are also malabsorbed. Systemic infections disturb virtually all normal metabolic and endocrine functions. These responses are not dependent on the infectious agent and involve both anabolism and catabolism.

However, the morbidity associated with multiple episodes of diarrheal diseases is increasingly recognized to have potentially devastating consequences. The diarrheal episodes may cause persistent intestinal injury leading to malabsorption and greater susceptibility to further infections. These sequelae may compromise the absorption of critical micronutrients for brain development like iron and vitamin B_6 (124), leading to irreversible structural and biochemical brain damage. The number of diarrheal episodes in the first two years of life has been shown not only to affect growth but also fitness, cognitive function, and school performance (91).

2.2 Cholera and its pathophysiology

Historically, cholera has been considered to be an ancient disease. There are references to death due to dehydrating diarrhea dating back to Hippocrates (ca. 460-370 B.C) and Sanskrit writings. Epidemic cholera was described at Goa, India in 1563 by Garcia del Huerto. The first cholera pandemic was reported to occur from 1817 to 1823 and spread from Indian subcontinent to other Asian countries. During second pandemic in 1830, it reached to western hemisphere. Afterwards, six more pandemics occurred in the world until 1993. *V. cholerae*, the causative agent of cholera has been serotyped on the basis of their LPS O antigens. The first six pandemics were caused by an O1 strain called the classical strain. The seventh pandemic introduced a new O1 strain called El tor, which had some different biochemical properties and phage susceptibility patterns compared with the classical strain. Eventually scientists discovered several strains of *V. cholerae* and found those are not as virulent as O1. In 1992, a newly described, non-O1 serogroup of *V. cholerae* designated O139 Bengal, caused an unusual cholera outbreaks in India and Bangladesh called the eighth pandemic. To date more than 200 serotypes of *V. cholerae* were discovered and among them O1 and O139 are now coexist and cause large outbreaks every year (174).

V. cholerae is a motile, gram negative, curved rod with a single polar flagellum. In 1884, Robert Koch successfully isolated the bacterium from the intestinal discharges of cholera patients and called it as "comma bacilli". It persists in the environment because it can survive in both fresh and brackish water. The mode of transmission of cholera is mainly by water and contaminated food. Although the infectious dose of *V. cholerae* is high enough (10^8/ml), studies have shown that a much lower dose (10^5/ml) is sufficient to cause infection if given with stomach acid neutralizers such as antacid. After an incubation period of between 18h and 5 days, symptoms are generally abrupt and include watery diarrhea and vomiting. The most distinctive feature of cholera is the painless purging of voluminous stools resembling rice-water. The term "rice water stool" has been used because the faecal stream becomes so dilute that it looks like water in which rice has been washed. In adults with severe cholera, the rate of diarrhea may quickly reach 500 – 1000 ml/h, leading to severe dehydration. Signs of severe dehydration include absence or low-volume peripheral pulse, undetectable blood pressure, poor skin turgor, sunken eyes, and wrinkled hands and feet (91).

The pathogenesis of cholera is relatively straightforward. Once the ingested vibrios are able to pass through the acidic stomach and colonize the upper small intestine by the help of toxin coregulated pili (tcp pili) that attach to receptors on the mucosa and by the bacterium's flagella. Concentrations of vibrios on the mucosal surface rapidly increase to 10^7 or 10^8 cells per g which efficiently deliver enterotoxin directly to the mucosal cells. The cholera enterotoxin has a molecular mass of 84 000 kDa and consists of five binding (B) subunits and one active (A) subunit. As we now understand the mechanism of action, the B subunits are physiologically inactive but bind the holotoxin to the GM1 ganglioside receptors in the small-intestinal mucosa, and the A subunit is transported into the cell where it activates adenylate cyclase. This activation leads to an increase in cyclic AMP, followed by an increase in chloride secretion in the crypt cells, and inhibition of neutral sodium chloride absorption in the villus cells. This in turn leads to a massive outpouring of fluid into the small intestine (174). The volume secreted exceeds the normal absorptive capacity of the bowel and results in watery diarrhea. The diarrheal fluid contains large amounts of sodium, chloride, bicarbonate, and potassium, but little protein or blood cells. The loss of electrolyte-rich isotonic fluid leads to blood volume depletion with low blood pressure and shock (174).

2.3 History of Oral Rehydration Solution

2.3.1 Oral rehydration therapy in cholera

The importance of oral rehydration therapy (ORT) is now globally recognized and its role in preventing the vast majority of deaths due to diarrhea or cholera is well established. It is evident from a quotation in *Lancet* in 1978, that elucidation of the biological mechanisms behind oral rehydration is "potentially the most important medical advance of this century" (63).

Clearly, before the scientific discovery of the mechanism by which ORT works, rehydration therapy was known and partially employed by using a variety of folk and medical mixtures and solutions. Empirically, it had become apparent in many places that one could ameliorate the general condition of a diarrhea patient by giving solutions containing salt and sugar or cereals or starchy vegetables (202). Dating from prescriptions by the Indian physician Sushruta some 3000 years ago, rice water, coconut juice and carrot soup have been widely

used throughout cholera's history. But only when the second pandemic hit Russia, dehydration was recognized as the cause of death due to cholera (91).

Before oral rehydration therapy was developed, intravenous fluid therapy was the main stay of fluid therapy for diarrheal dehydration. I.V fluid therapy was first attempted in Russia and Scotland in 1830. It was Sir Leonardo Roger who introduced hypertonic saline in 1890 and reduced mortality of hospitalized cholera cases from 61% to 33% (4). Further improvement of I.V. therapy was done by Andrew in 1909 and Daniel Darrow in 1940 by adding bicarbonate and potassium respectively (189). The cotransport of the sodium ion across the intestinal mucosal epithelium, from the lumen into the "milieu interieur," was defined during the 1950s principally in the laboratories of physiology at Oxford University and biophysics at Harvard University. Knowledge of this cotransport process made it possible to begin to develop a practical treatment for cholera (202). In 1961, Phillips and Wallace demonstrated that patients with cholera drank sodium chloride solution but did not absorb the salt, and that when glucose was mixed in oral fluids, it was fully absorbed and enhanced sodium absorption (91). At the same time, Gangarosa *et. al.* showed that no epithelial cell damage occurs in gut in cholera (189). Afterwards, the efficacy of glucose-ORS was demonstrated in several laboratories and countries in many different situations. It was proved so effective that, when an appropriate amount is given orally to a child or adult with signs of dehydration resulting from acute watery diarrhea, rehydration can be accomplished in 4-8 hours (48, 62). During the liberation war of Bangladesh in 1971 Dr. Dilip Mahalanabis showed efficacy of glucose-ORS in cholera cases amongst Bangladeshi refugees who took shelter in the border of Bangladesh and India. Dr. Sircar *et. al* demonstrated the efficacy of ORS in a cholera epidemic in Manipur in 1978. Based on all beneficial and life saving information of glucose-ORS around the world, the World Health Organization in 1978 launched global diarrheal diseases control program with ORS as the pivot with the short term objective of reduction of mortality due to diarrhea and cholera (189).

The composition of glucose-ORSs in different parts of the world designed afterwards to replace stool losses was generally based on that of electrolyte losses in diarrheal stools. In developing countries, the reference was cholera, whereas in North American and European countries, it was rotavirus infection (89). The composition of different ORSs are presented in table 1 (54).

Table 1. Recommended composition of an oral rehydration solution

Composition (mmols/l)	WHO*	AAP†	ESPGAN‡	RESOMAL§
Glucose	110	110-140	60	125
Sodium	90	45-75	60	45
Potassium	20	20	20	40
Citrate$^{\beta}$	10	7-10	3	7
Chloride	80	35-65	70	70
Magnesium				3
Zinc				300µmol
Copper				45µmol

* World Health Organization
† American Academy of Paediatrics
‡ European Society for Paediatric Gastroenterology and Nutrition
§ For malnourished children
$^{\beta}$ Citrate is used a trisodium salt, which corresponds to 30 mEq or 10mmols/1 in the WHO solution

2.3.2 Electrolyte transport mechanism of glucose-ORS in small intestine

The four basic ions e.g., Na^+, K^+, Cl^- and HCO_3^- transport from intestinal lumen to epithelial cell is one of the key functions of intestinal tract. These actively transported ions are primarily involved in controlling of fluid movement. Fluid follows the direction of electrolyte movement to maintain isotonicity between the intestinal lumen and tissue compartments. Intestinal mucosa, which surrounds the lumen of the intestinal tract, mediates the wide varieties of absorptive and secretory processes exhibited by the intestine. All of these processes depend essentially on the transport properties of the apical (or brush border) and basolateral membranes of these cells, and these transport properties are modulated by extracellular and intracellular factors and by ionic permeabilities of the lateral intercellular spaces (131).

There are specialized proteins associated with plasma membrane that can control movement of ions. The transmembrane proteins, located at the luminal basolateral side of the enterocytes, enable ions to move across the plasma membranes of an epithelial cell. Several transmembrane proteins has been described : SGLT1 for the Na^+-glucose co-transporter, CFTR for Cl^- channel, BSC for Na^+ , K^+, $2Cl^-$ cotransporter and NHE3 for Na^+/H^+ exchanger. A Na^+/glucose co-transporter exists on the apical membrane of the intestinal tract. It catalyses the co-transport of two Na^+ and one glucose, and thus involves the movement of two positive charges for each molecule of glucose transported (66). In this system, the energy is provided

by the activity of Na^+, K-ATPase pump. The glucose mediated sodium transport remains intact during diarrhea (Figure 1).

Chloride (Cl^-) secretion is enhanced in diarrheal condition due to the disturbance in enterocyte transport system. In this process, the cell takes up Cl^- from the blood stream

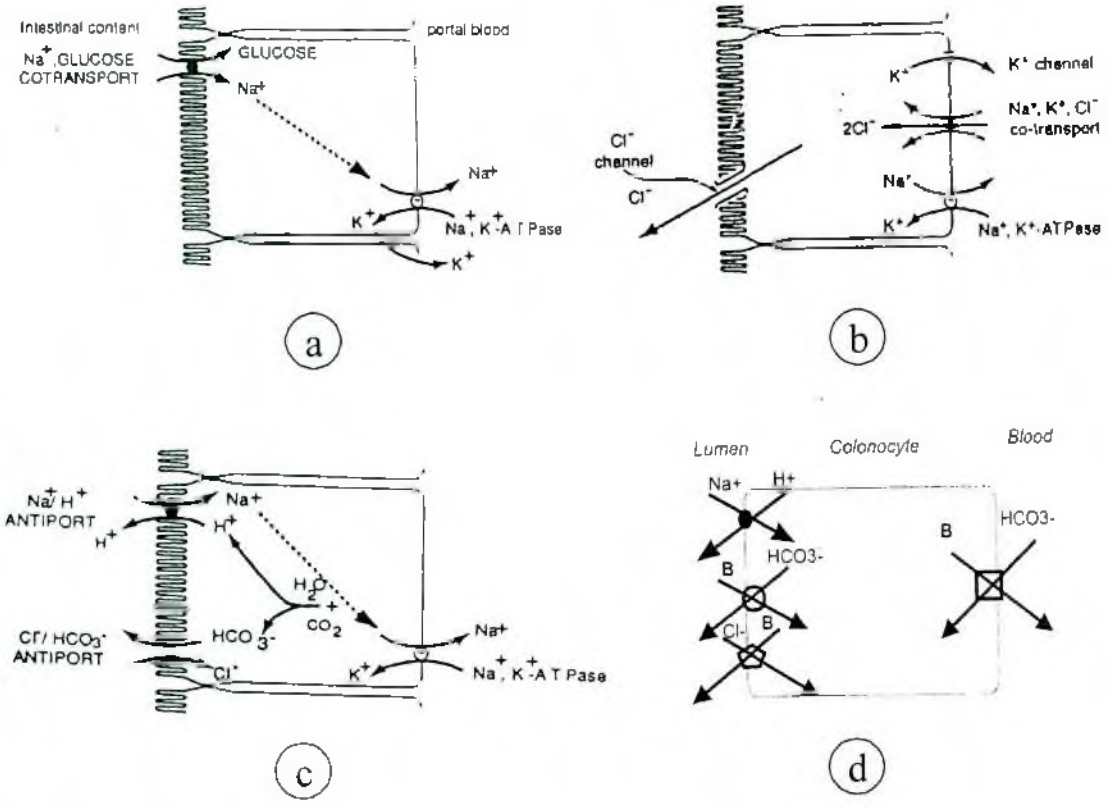


Figure 1. The four main electrolyte transport systems involved in diarrhea.

The sodium absorption is stimulated by glucose and amino acids, in the small intestine (a), and by SCFAs, in the colon (d). The stimulation of chloride secretion (b) is associated with a decreased NaCl absorption (c). Adapted from Binder and Mehta, 1989 (20).

across the basolateral membrane by way of the Na^+, K^+, $2Cl^-$ co-transport pathway and Na^+/K^+ ATPase provides energy for this mechanism. Cl^- accumulates intracellularly above its electrochemical gradient. Chloride channels remain usually closed in cell but open this time and Cl^- goes out across the apical membrane to the lumen. In the overall secretory mechanism, Cl^- is actively secreted transcellularly and Na^+, K^+ and water follow passively by way of the intercellular tight junction (Figure 1).

The third mechanism of sodium (Na^+) and Cl^- transport is composed of a Na^+/H^+ exchange working in parallel with a Cl^-/HCO_3^- exchange pathway in apical membrane. In a dual-exchanger model coupling of these paired exchangers occurs through changes in intracellular

pH. The Na^+ gradient drives Na^+ uptake and H^+ efflux by means of Na^+/H^+ exchange, which alkalinizes the cytoplasm and increases activity of the Cl^-/HCO_3^- exchanger. The action of carbonic anhydrase produces HCO_3^-, which then leaves the cell in exchange for uptake of luminal Cl^-. This mechanism allows both Na^+ and Cl^- to enter the cell in exchange for H^+ and HCO_3^- (Figure 1).

2.3.3 Electrolyte transport mechanism by SCFAs in large intestine

Studies have shown that absorption of water and electrolytes can be enhanced by short chain fatty acids in the colon (14, 52). The mechanism by which SCFA stimulate water and NaCl absorption in human colon, has been extensively studied by Binder *et al.* in rabbit colon for butyrate (155). Butyrate is the most effective of the three abundant SCFAs in stimulating sodium absorption. Butyrate stimulated active sodium and chloride absorption involves uptake of the non-ionized form of butyrate through two exchangers present in the luminal membrane of the colonocytes. They are sodium-hydrogen exchanger and chloride-butyrate exchanger. Once in the colonocyte, butyrate is partly absorbed toward the blood circulation by another transporter and partly recycled toward the luminal content through a butyrate-chloride antiport. As a result butyrate is absorbed across the epithelial layer of the colon together with NaCl, which drags water from the luminal content to the blood circulation (Figure 1) (53).

2.3.4 Limitations of Glucose-ORS

The promotion of oral rehydration therapy by WHO has been extremely successful. Most families, primary health care centres and university hospitals all over the world are now aware of the risk of dehydration in watery diarrhea and of the efficacy of oral rehydration therapy. At the same time, however, its limitations also became apparent. The primary limitations of glucose-ORS can be summarized as follows: a) it does not reduce the stool volume and if given in excess, it may even increase the volume of watery stools, b) it does not shorten the duration of diarrhea, c) it does not minimize the fluid requirements and d) the

beneficial effect on nutritional status has been questioned. By itself, 20 g of glucose per litre of ORS did not constitute a significant source of energy. Moreover, simply by increasing the concentration of glucose in ORS, its efficiency cannot be enhanced (55).

2.3.5 Approaches to improve the ORS

The choice of solutes to improve ORS has been based on physiological considerations. The small intestine is the organ through which the largest amounts of water and electrolytes are transported daily (55); it is also the site of absorption of organic solutes e.g., glucose and amino acids. Na^+ absorption is stimulated by the presence of solute at the luminal membrane which is directly related to its molecular structure and not to the extra energy it may supply through its metabolism. For example, glucose does not stimulate Na^+ absorption by supplying a surplus of ATP to the Na^+ - K^+ ATPase on the basolateral side of the enterocytes (87), but rather by binding to the cotransporter $SGLT_1$ present on the luminal membrane. Although most water soluble solutes are probably transported with Na^+, only monosaccharides and amino acids are present in the intestinal lumen in sufficient amounts to stimulate Na^+ absorption via a cotransport mechanism. Of the monosaccharides, both glucose and galactose (but not fructose) stimulates Na^+ absorption, and amino acids and some dipeptides are also directly coupled to Na^+ absorption. The efficacy of amino acids in stimulating Na^+ absorption depends on the pH of these amino acids. The basic or cationic amino acids are poor stimulators of Na^+ absorption, and the acidic amino acids stimulate it more than the neutral amino acids. Neutral amino acids stimulate Na^+ absorption as effectively as glucose. Among the amino acids, glycine, alanine, glutamine and the di-peptide alanyl-glutamine demonstrated enhanced water absorption in some studies (182).

The concentration of candidate solutes is another important factor which is based on the osmolality of the solution. Physiological studies showed that the small intestine has a leaky epithelium that cannot absorb water and electrolytes against a steep osmotic gradient between lumen and blood. The upper limit of osmolality at which intestinal absorption is reduced to zero is ~ 400 mOsm/L in the lumen compared with 300 mOsm/L in the blood. Therefore, the

choice of substrate concentration was designed to reach a final osmolality of ≤300 mOsm/L of ORS (55).

These physiological considerations were justified by the finding that many traditional solutions used to treat diarrhea (e.g., rice-water) have a low osmolality (frequently ≤150 mOsm/L). Replacement of glucose with sucrose, which supplies an equal amount of glucose, yields an ORS as effective as standard glucose-ORS (138, 147, 172, 173). Again, replacement of glucose by brown sugar molasses also does not hamper the rehydration efficacy of ORS (99). However, no additional clinical advantages were found when maltodextrins, amino acids, or dipeptides were used instead of glucose (55).

To increase the universal acceptance, availability and food-like character of ORS, cereals were included in its composition as a source of glucose. Various cereals have been tested as ORS substrates including rice, maize, millet, sorghum and wheat (4, 19, 88). The general conclusion of these studies is that cereal-based ORS are as safe and effective as glucose ORS in rehydrating children with diarrhea with diverse etiologies even in cases of severe malnutrition. The first clinical trials comparing rice–ORS with glucose-ORS demonstrated the superiority of rice-ORS in terms of shortening the duration of diarrhea and reducing the volume of stools (129, 150). However, with more studies being conducted, the difference became less obvious. In this regard, the meta analysis by Gore *et. al.*, 1992 was of particular interest because it was based on the data from 13 clinical trials in which the effect of rice and glucose based ORS were compared (84). The results indicated that stool output was reduced in higher magnitude by rice ORS in cholera (36% in adults, 32% in children), but in lower magnitude in non cholera diarrhea (18% in infants and children).

While fluid secretion in human cholera is thought to occur predominantly from the small intestine, recent data suggest that water absorption from the colon is reduced or that the colon may even be in a secretory state. Ramakrishna *et al.*, showed that the concentration of short chain fatty acids (SCFA) in faeces in patients with cholera is reduced and that the addition of luminal SCFA can stimulate the colonic absorption of salt and water (156). So, an alternative approach for treatment of dehydration due to cholera has been suggested adding amylase

resistant starch in glucose ORS. The rationale for using amylase resistant starch was that in the colon starch is fermented by anaerobic bacteria and produces the short chain fatty acids e.g. acetate (C2), propionate (C3), and butyrate (C4). The main reason for using resistant starch for rehydration is to take advantage of the ability of these SCFA to enhance water and electrolyte absorption in the colon (6, 23, 158). Contrary to the previous studies aimed at improving rehydration, the use of resistant starch is an attempt to add the absorptive functions of the colon to the already used functions of the small intestine. The general hypothesis is that short chain fatty acids may be used in the colon to promote water and electrolyte absorption just as glucose does in the small intestine.

2.4 The human gut microflora

2.4.1 Overview of the human digestive system

The digestive system includes the digestive tract and its accessory organs, which process food into molecules that can be absorbed and utilized by the cells of the body. The digestive tract, also called the alimentary canal or gastrointestinal (GI) tract, consists of a long continuous tube that extends from the mouth to the anus. In humans over 5 years old the GI tract is 6.5 m to 9.0 m long. It includes the mouth, pharynx, esophagus, stomach, small intestine (duodenum, jejunum and ileum), caecum, large intestine or colon (ascending, transverse, descending and sigmoid colon), rectum and anus (Figure 2). The tongue and teeth are accessory structures located in the mouth. The salivary glands, liver, gallbladder and pancreas are major accessory organs that have a role in digestion. Although the small intestine is much longer than the large intestine (typically 4-5 times longer) it is referred to as such due to its comparatively smaller diameter. On average, the diameter of the small intestine of an adult human measures approximately 2.5-3 cm and the large intestine measures about 7.6 cm.

The digestive tract, from the esophagus to the anus, is consists of a wall with four layers, from the inside of the tract to the outside they are: i) mucosa, ii) submucosa, iii) muscle layer and iv) serosa (Figure 3). Depending upon the section of the digestive tract, mucosa protects the GI tract wall, secretes substances, and absorbs the end products of digestion. It is composed of a layer of epithelial cell and lamina propria. The submucosa lies outside the mucosa. It consists of areolar connective tissue containing blood vessels, lymphatic

vessels, and nerve fibres. The next layer corresponds to smooth muscles comprised of circular fibres surrounded by

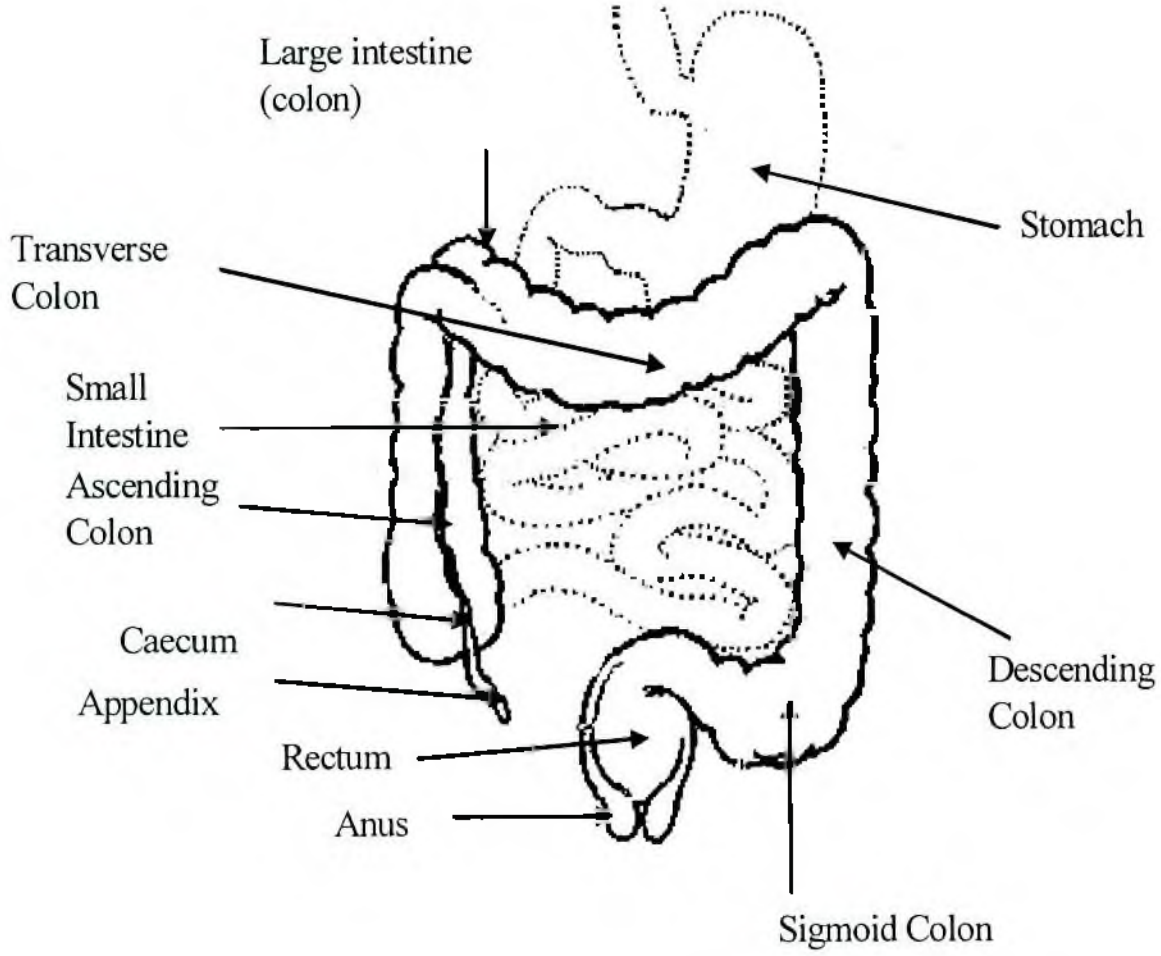


Figure 2. A detail view of the human digestive tract.

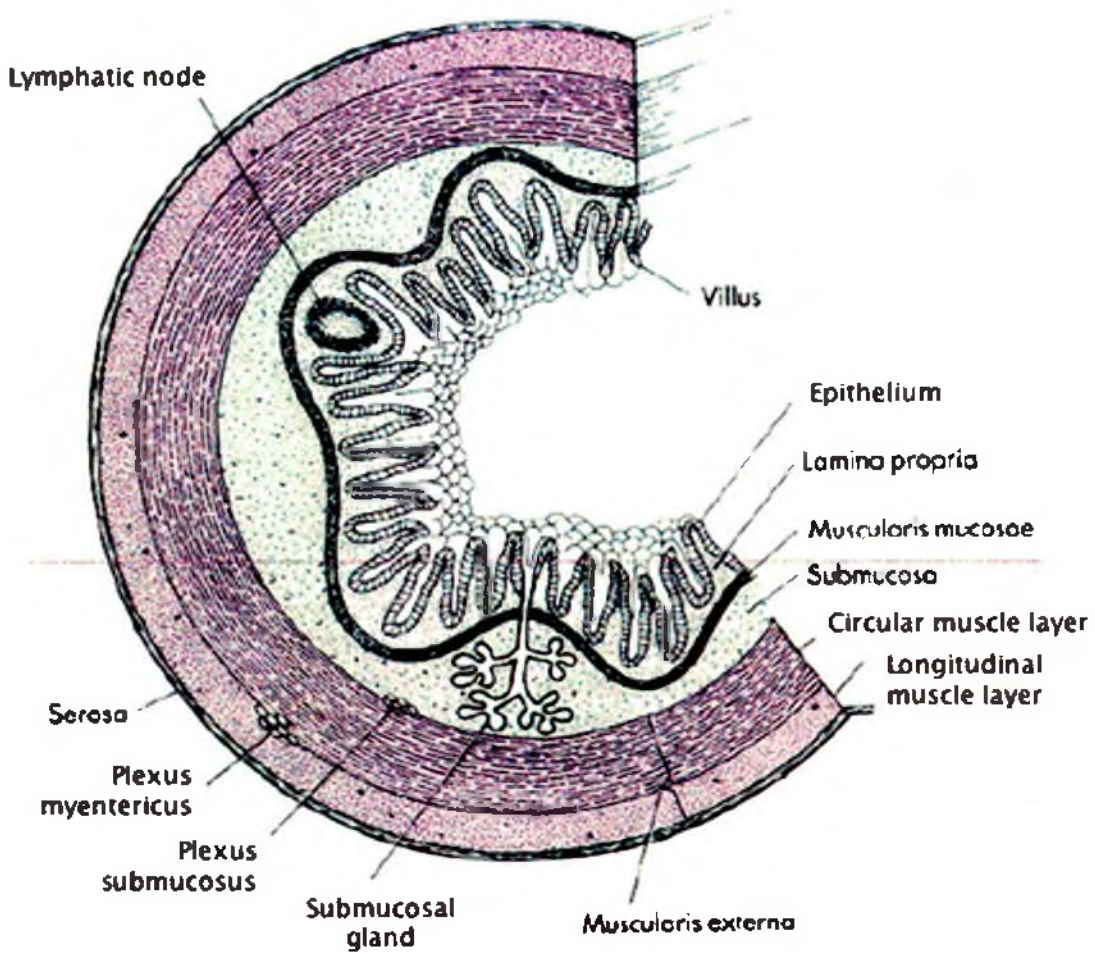


Figure 3. Layers of gastrointestinal wall.

longitudinal fibres and the final layer serosa consist of secretory epithelial layer and connective tissue layer.

Digestive system has four types of activities to digest the food: i) Ingestion and movement of the food, ii) Secretion of gastric juices and digestion, iii) Absorption of nutrients and iv) Elimination.

- Ingestion and movement of the food

  The first activity of the digestive system is to take in food through the mouth. This process, called ingestion, has to take place before anything else can happen. The large pieces of food that are ingested have to be broken into smaller particles that can be acted upon by various enzymes. This is mechanical digestion, which begins in the mouth with chewing or mastication and continues with churning and mixing actions in the stomach. After ingestion and mastication, the food particles move from the mouth into the pharynx, then into the esophagus. Food moves from one organ to the next through muscle action called peristalsis. Peristalsis looks like an ocean wave traveling through the muscle. The muscle of the organ contracts to create a narrowing and then propels the narrowed portion slowly down the length of the organ. These waves of narrowing push the food and fluid in front of them through each hollow organ.

- Secretion of gastric juices and digestion of foods

  The digestive glands that act first are in the mouth—the salivary glands. Saliva produced by these glands contains amylase that begins to digest the starch from food into smaller molecules. The next set of digestive glands is in the stomach lining. They produce acids/and an enzymes in stomach that digests protein. Stomach empties the food and juice mixture into the small intestine, and in small intestine the juices of two other digestive organs mix with the food. One of these organs, the pancreas, produces a juice that contains a wide array of enzymes to break down the carbohydrate, fat, and protein in food. Other enzymes that are active in the process come from glands in the wall of the intestine. The second organ, the liver, produces yet another digestive juice, bile. Bile is stored in the gallbladder between meals. During meal, it squeezed out of the gallbladder into intestine through the bile ducts. Bile mixes with the fat in

intestine. The bile acids dissolve fat into the watery contents of the intestine and are further digested by pancreatic and other enzymes.

- Absorption of nutrients

  Most digested molecules of food (carbohydrate, protein, fat, vitamins), as well as water and minerals, are absorbed through the small intestine. The mucosa of the small intestine contains many folds that are covered with tiny fingerlike projections called villi. In turn, the villi are covered with microscopic projections called microvilli. These structures create a vast surface area (approx.180 m^2) through which nutrients can be absorbed. Specialized cells allow absorbed materials to cross the mucosa into the blood, where they are carried off in the bloodstream to other parts of the body for storage or further chemical change.

- Elimination

  The food molecules that cannot be digested or absorbed need to be eliminated from the body. The removal of indigestible wastes through the anus, in the form of faeces, is defecation or elimination.

2.4.2 Microbial world in the digestive system

Although the Gastrointestinal tract is a major barrier to the penetration of microorganisms into the body, it also provides a suitable environment for microbial growth. Microorganisms are not equally distributed in the GI tract. Along the tract, they are generally found in the ecological niches where physiological conditions (e.g., pH) are favorable for their growth. In the stomach the pH is highly acidic (1 to 3) and only a small number (10^3 to 10^4 per g) of bacteria are found; these includes and mostly *Helicobacter pylori*, *Lactobacillus*, *Streptococcus* and some yeasts are present. As bile and pancreatic juice are added to duodenum the acidic environment is turned to be alkaline in lower part of small

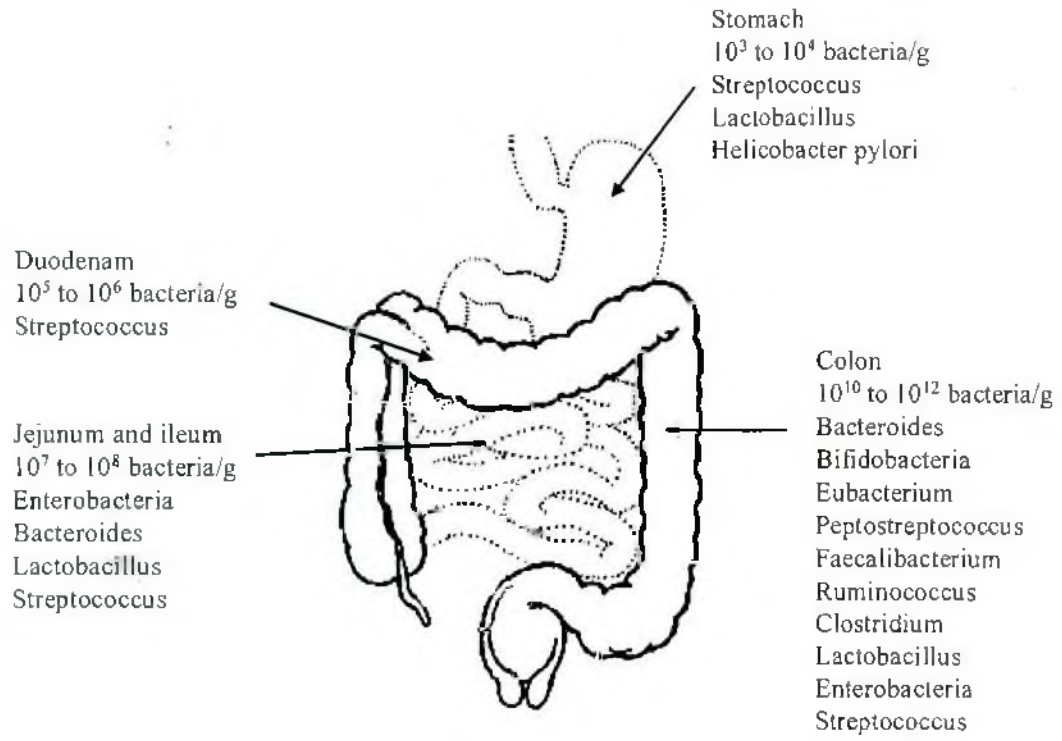


Figure 4. Distribution of microorganisms in digestive tract.

intestine and bacterial numbers increase. The duodenum contains a bit higher bacteria: 10^5 to 10^6 per g with predominantly the genus *Streptococcus*. In the jejunum and ileum bacterial populations reach 10^7 to 10^8 bacteria per g and *Streptococcus* as well as *Lactobacillus, Enterobacteria, Bacteroides* are found. The large intestine, or the organ which is now more commonly referred to by its Greek name, the 'colon' is the last part of the digestive system. In the colon the strictly anaerobic bacteria become dominant over the aerobic and facultative anaerobic bacteria by a factor of 10^2 to 10^4 and reach the concentration of 10^{10} to 10^{12} bacteria per g (187). The predominant species are of the following genera: *Bacteroides, Bifidobacterium, Eubacterium, Peptostreptococcus, Faecalibacterium, Ruminococcus, Clostridium, Lactobacillus, Streptococcus and Enterobacteria* (Figure 4). Due to sampling difficulty in large intestine, the faecal flora are usually studied to learn about the microflora of colon (73). A large proportion of the faecal mass (dry weight) consists of bacteria, approximately 60% of faecal solids (90).

The intestine is adapted to a bi-directional host ↔ microflora exchange and harbours a diverse bacterial community that is separated from the internal milieu by only a single layer

of epithelial cells (143). Under normal conditions, the gut microbiota is contained within the lumen of the gut with minimal invasion of the mucosal layer. Bacterial growth and activities are higher in the right than the left colon as the luminal contents enter first in the right part from the small intestine. Colonic transit time is another important factor for bacterial activities because the higher the transit time, the microbiota get more time to metabolize the partially digested nutrients and at the same time absorption of nutrients, water and electrolytes are higher. In an healthy individual the gut transit time ranges from 35 to 125 hr with an average of 61hr. Stool volume and excretion of bacterial biomass increase with the faster transit time in gut (194).

Although the large intestine is colonized by a dense and complex community of microbiota they are not floating in/on the digesta rather the bacteria exist in a multiplicity of different microhabitats and metabolic niches that are associated with the mucosa, the mucus layer and particle surfaces in the colonic lumen. Bacteria in the large bowel may occur individually, but it is more likely they exist in microcolonies or in associations with other species on the surfaces of particulate materials forming bio-films on it. Bacteria growing in bio-films often behave differently from their non-adherent counterparts, and in particular, the nature and efficiency of their metabolism is changed, while many species exhibit greater resistance to antibiotics, and other inhibitory factors that have deleterious effects on planktonic bacteria (13). Biofilm communities often exhibit highly coordinated multicellular behaviors, within and between species, and many biofilm properties are dependent on local cell population densities. A good example of this is provided by quorum sensing transcriptional activation in Gram-negative bacteria (177). Mucosal populations are difficult to study in healthy people, despite this, some reports suggest that gut epithelial populations in man are generally similar to those present in the gut lumen (139). *Bacteroides sp.* occur on the mucosal surface, but other gut anaerobes including *Eubacteria, Bifidobacteria, Clostridia* and a variety of Gram-positive cocci are also present (42).

2.4.3 Diversity in the adult faecal microbiota

Studies performed during the past three decades have given an approximation of the complexity of the normal colonic micro flora. Several major approaches based on aerobic and

anaerobic culture techniques were adopted in different laboratories around the globe to study the colonic flora. Moore and Holdeman, 1974 cultivated faecal samples of 20 male healthy Japanese-Hawaiians, aged from 60 to 80 years, using non selective medium containing rumen fluid in roll-tubes under anaerobic condition maintained by CO_2 gas (133). They could isolate 1147 different colonies (44 to 69 per stool) and identified 113 different types of bacteria among them. Statistical estimates indicate that these types account for 94% of the viable cells in the faeces. According to statistical analysis by Good *et al.*, they estimated that the total number of different kinds of bacteria in the intestinal tract at any given time probably exceeds 400 or 500 species, but many are represented by less than 10^8 cells per g of faeces. Direct microscopic clump counts indicated that the mean total flora was 5.06 x 10^{11}bacteria per g of faeces (dry matter) and ranged between 6.9 x $10^{10}$and 1.43 x 10^{12}. The mean culture count was 4.75 x 10^{11} cfu per g of faeces (dry matter) and varied from 1.8 x 10^{11} to 1.36 x 10^{12}. The predominant bacterial species includes *Bifidobacterium adolescentis, Bacteroides vulgatus, Collinsella aerofaciens, Faecalibacterium prausnitzii, Ruminococcus productus, Bacteroides thetaiotaomicron, Eubacterium biforme, Eubacterium rectale and Bacteroides distasonis*. Mean counts of Clostridium and facultative anaerobes were lower (10^8 to 10^9 colonies per g).

By using modified medium 10 in combination with strict anaerobic technique Mitsuoka *et al.*, 1976 studied the faecal flora of 42 healthy adult males (127). The bacterial genera frequently retrieved were *Bifidobacteria, Catenabacteria (Eubacteria* and *anaerobic Lactobacilli), Peptostreptococci, Clostridia, spirillaceae, Veillonellae* and *Megasphaerae.* The total bacterial count of faeces from healthy adult male was 1-2 x 10^{11} per g wet weight. Another major approach to the analysis of faecal flora was taken by Finegold *et al.*, 1983 who analysed 142 faecal samples on selective media in an anaerobic chamber (73). Of those 142 subjects 13 volunteers were strictly vegetarians, 14 ate small amount of meat, 15 were on a Japanese diet, 25 patients with colonic polyps, 12 had colon cancer and 62 volunteers were on a normal western diet. The average bacterial count was 1.58 x 10^{12} cfu per g of faeces (dry matter). The dominant species retrieved were the same as mentioned earlier except *F. prausnitzi*. They also obtained three species frequently e.g., *Eubacterium contortum, Eubacterium cylindroids* and *Eggerthella lenta.*

This chapter follows by a brief description of some dominant bacteria frequently obtained by culture methods in human faeces. The *Bacteroides* are the most numerous members of the

normal flora. Being obligate anaerobic, gram-negative, pleomorphic rods they accounted for 20 to 30% of total cultivated flora in human faeces (133). The frequently found species are *Bacteroides thetaiotaomicron, B. vulgatus, B. distasonis, B. fragilis,* and *B. ovatus.* Formerly, the above-mentioned organisms were all classified as subspecies of *Bacteroides fragilis;* however, DNA homology studies indicate that these organisms should reported as separate species (104). They could be detected in more than 99% of the faecal samples and the total bacteroides counts ranged from 1.58×10^9 to 3.16×10^{13} with an average of 1.99×10^{11} cfu per g (73).

Anaerobic cocci were isolated from the stool in high counts: range from 10^4 to 2.5×10^{13} colonies per g; (mean count 5.0×10^{10} colonies per g). *Peptococcus, Peptostreptococcus, Ruminococcus, Coprococcus* and anaerobic streptococci were among the groups commonly isolated. They are Gram-positive, non-spore forming and chemoorganotrophic (96). Peptococcus species was isolated from 10% of human stools, *Peptostreptococcus* from 45.4%, *Ruminococcus* from 45%, *Coprococcus* from 5.7% and anaerobic streptococci from 99.3% of human faecal samples (73) .

The most common Gram negative anaerobic cocci are from the genera of *Veillonella, Acidaminococcus and Megasphaera.* They are nonsporeforming, nonmotile, oxidase and catalase negative. Members of the *Veillonellae* transform lactate into acetate, propionate, CO_2 and H_2. They are chemoorganotroph and usually produce significant amounts of gases (165). The average counts of *Veillonella* were 7.9×10^7 and were detected in 34% of samples. *Acidaminococcus fermentans* and *Megasphaera elsdenii* were isolated from 19% and 5% samples and their average counts were 3.2×10^8 per g and 2.5×10^9 per g, respectively.

The genus Eubacterium consists of a diverse collection of Gram-positive, nonsporing, obligate anaerobic, chemoorganotrophic and rod shaped bacteria. The frequently isolated species includes *Eubacterium biforme, Eubacterium contortum, Eubacterium cylindroids, Eubacterium eligens, Eubacterium formicigenerans, Eubacterium rectale* and *Eubacterium ventriosum* (73, 133). Among these, two dominant faecal Eubacterium have recently been reclassified: *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*) and *Eggerthella lenta* (formerly *Eubacterium lentum*). In the study of Finegold *et al.* they were the most frequent bacteria of the Eubacterium genus. *Eubacterium sp.* could be detected from 94% of the human faeces with an average count of 5.01×10^{10} (73) .

The major human intestinal *Bifidobacterium sp.* were *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum* and was detected in 74% of adult human stools (73). They are Gram-positive, non spore forming, strictly anaerobic, non-motile and displays a variety of different shapes e.g., spatulated extremities, star like aggregates, middle enlarged cells, long chains of regular rods, V or palisade arrangements and amphora-like cells (180). The average numbers of Bifidobacterium sp. in human faeces were 1.58×10^{10} per g (73). Colonization by *Bifidobacteria* was found to be significantly higher in breast-fed babies (47.6% of breast feed babies vs. 15% bottle fed babies) (168).

Clostridium species are generally Gram-positive, spore forming, strictly anaerobic bacteria. This species sometimes stain Gram negative specially if the culture is old. Clostridium species establish a low redox potential (-400 to 200 mV) in its habitat. Growth can not occur if redox potential is above 150 mV (86). The most frequent faecal species includes *C. bifermentans, C. innocuum, C. nexile, C. paraputrificum, C. perfringens, C. ramosum, C. sporosphaeroides*. They were isolated from all healthy and diseased human faeces by Finegold *et al.* and the average count was 6.31×10^9 cfu per g (73).

Lactobacilli are also prominent adult intestinal flora. In the Wadsworth study, *Lactobacillus* was isolated from 78.0% of human faeces, with a mean count of 3.9×10^9 cfu per g. The most common isolate, *L. acidophilus,* was also the most common species seen by Moore and Holdeman, 1974 (133). *Lactobacillus* is a genus of Gram-positive, non spore forming, facultative anaerobic or microaerophilic bacteria. They are a major part of the lactic acid producing bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid. They may be either homofermentative (end product is lactate) or heterofermentative (end product is lactate, CO_2, ethanol, and /or acetate). The frequently found species are *L. fermentum, L. leichmannii, L. plantarum, L. salivarius, L. brevis, L. casei, L. lactis*.

Faecalibacterium (Formerly Fusobacterium) species are obligatory anaerobic, Gram-negative, non motile and non-sporforming rod shaped bacteria. They metabolize peptone and carbohydrates and produces Butyrate (132). *Faecalibacterium prausnitzi* was the second most abundant organism in human faeces (133). Other frequently retrieved species are *F. gonidiaformans, F. mortiferum, F. necrophorum, F. russii*. The mean count of these bacteria

in human faeces was 2.51 x 10^8 cfu per g and they were isolated from 18.4% of the human samples but neither in vegetarian subjects nor patients with colon cancer (73).

Members of the Enterobacteriaceae family are another most common species found in human faeces. They are facultative anaerobic, Gram negative, straight rods. Their metabolism is either respiratory or fermentative and they are chemoorganotrophic. Generally they are oxidase-negative and catalase positive (30). The major human intestinal species retrieved were *Escherichia coli, Citrobacter freundii, Enterobacter cloacae, Hafnia alvei, Klebsiella pneumoniae and Serratia liquefaciens* (73, 133). In case of being opportunistic pathogen, some members of this genus occasionally cause gastrointestinal diseases e.g., *Shigella, Salmonella, E. coli* and *Yersinia.* The average number of these bacteria was 5.01 x 10^8 cfu per g of faeces and they could be retrieved from 98% of the human subjects (73).

2.4.4 Diversity of microflora in healthy children

The foetal intestine is sterile and bathed in swallowed amniotic fluid. Following delivery, multiple different antigens challenge the intestine of the newborn. During passage through the birth canal the infant is exposed to the maternal indigenous flora (2). However, independent of the source, at day 1 the neonatal intestine is first colonized with *Enterobacteria*, whose number attains 10^9 cfu/g faeces (220). In the subsequent few days, bacteria multiply in the gut under the influence of the individual enteric environment, the bacterial interactions and, especially, the infant diet. According to Sakata *et al.*, on the first day of life, *Enterobacteria, Streptococci, Enterococci* and *Staphylococci* were already present; anaerobes such as *Bifidobacteria*, *Lactobacilli* and *Bacteroides sp.* were usually not found (176). The gradual consumption of oxygen in the intestine by aerobic microorganisms decreases the oxidation-reduction potential and thus provides the conditions for the settlement of anaerobic bacteria (146). At the end of the first week, a diet of breast milk creates an environment favoring *Bifidobacteria.* Yoshioka *et al.* investigated the development of the faecal flora in 6 breastfed newborns and found that, in breastfed neonates by day 6 *Bifidobacteria* exceeds *Enterobacteria* by a ratio of 1000/1 (220).

At one month of age, the colonic flora were stable in the majority of breastfed babies and dominated by *Bifidobacteria*, besides this they also harbors *Enterococci, Enterobacteria* and *Clostridia* (17, 220). However, by 3 month of age the predominance of *Bifidobacteria* was slightly reduced; but remain numerous and existence of *Enterobacteria, Enterococci, Staphylococci, Bacteroides, Lactobacilli* and *Clostridia* were also reported (162). After weaning the faecal flora comes to resemble close to that of adult flora. The frequency and numbers of *Bacteroides* and anaerobic Gram-positive cocci continue to increase until match to *Bifidobacterium. E. coli* and *Streptococcus species* decline to about 10^6 to 10^8 CFU per g of faeces. By the second year of life major bacterial populations in faecal flora come to resemble those of adults (193).

Albert *et al.*, 1978 cultivated faecal samples of 29 healthy children of Southern India, aged from 1 to 20 months, using non selective and selective medium under aerobic and anaerobic condition (8). The children were from the lower socio-economic strata and none had had received any antimicrobial drug before the study. The younger children were on breast-milk feeds and often supplemented with cow's milk. The older children were also receiving cow's milk in addition to weaning foods. They could retrieve anaerobic bacteria predominantly with, on average, 126 anaerobes to each aerobe. *Bifidobacteria* were invariably found and gave rise to a mean count higher than that produced by any other organism. The bulk of the aerobic flora consisted of *Enterobacteria* and *Enterococci.* The viable count of the microflora is presented in Table 2 and 3.

In another study, Ellis-Pegler *et al.*, 1975 collected faecal samples from 12 children from South London, England aged from 13 to 48 months (mean 25.3 months) and studied by aerobic and anaerobic culture (65). None of the children had a history of abnormal bowel function or of antibiotic therapy within the preceding months. No infants in the study had been breast-fed. Most were fed on diets which includes a wide range of commercial milk food preparations as the predominant component. The investigators observed that, the ratio of total anaerobes: total aerobes were approximately 50:1. Viable count of *Enterobacteria, Staphylococci, Streptococci, Lactobacilli, Bacteroides, Bifidobacteria, Clostridia* and *Veillonella* were performed in this study and presented in Table 2 and 3.

Faecal microflora was also analyzed by Mata *et al.,* 1972 among 12 healthy infants of Guatemala aged 9 to 12 months, using aerobic and anaerobic culture (123). The faeces were

cultured fortnightly and a total of 262 specimens were analyzed. The frequently obtained bacterial species were *Bifidobacteria, Veillonellae, Streptococci, Bacteroides, Micrococci, Enterobacteriaceae* and *Enterococci* where, *Lactobacilli and Clostridia* were relatively rare. Viable count of Bifidobacteria was higher than any other group of bacteria. The Bacterial number is presented in Table 2 and 3.

Table 2. Viable count (Log10/g) of facultative anaerobic bacterial groups in faecal samples of children.

					Facultative anaerobic bacteria (%)	
Studies	Ref No.	Location	N	Age (Months)	*Enterobacteria*	*Enterococcus sp.*
Mata *et al.*	123	Guatemala	12	9-12	6	6
Albert *et al*	8	South India	29	1-20	8.3	7.8
Ellis-Pegler *et al.*	65	South London	12	13-48	8.3	ND

ND – Not done

Table 3. Viable count (Log10/g) of anaerobic bacterial groups in faecal samples of children.

					Anaerobic bacteria (%)					
Studies	Ref No.	Location	N	Age (Month)	Bif	Bact.	Clos	Staph	Lacto	Veillo
Mata *et al.*	123	Guatemala	12	9-12	9	7	8	ND	8	8
Albert *et al.*	8	South India	29	1-20	10.1	8.8	5.2	3.5	6.9	8.4
Ellis-Pegler *et al.*	65	South London	12	13-48	9.1	10	7.5	3.6	3.5	7

Bif, *Bifidobacterium sp.*; Bact, *Bacteroides sp*; Clos, *Clostridium sp*; Staph, *Staphylococcus sp.*.; Lacto, *Lactobacillus sp.*; Veillo, *Veillonellacae sp.*

2.4.5 Factors influencing the diversity of faecal microbiota in children

Several factors influence the diversity and composition of human intestinal microflora among them malnutrition, disease process and antibiotics have more profound effect on this ecosystem. They are discussed below:

2.4.5.1 Malnutrition

Malnutrition especially protein-calorie malnutrition is the principal nutritional problem in most developing countries of the world. Malabsorption is observed in children with severe malnutrition. Severely malnourished children are frequently presented with diarrhea and have an altered gastrointestinal function. Mata *et al.* 1972, studied faecal microflora from 13 severely malnourished children both by aerobic and anaerobic condition by roll tube technique and plate dilution technique (121). After enrolment in the study, the subjects were placed on a diet similar to the one they received in their homes for 1 or 2 days. Aspirates were obtained from the stomach, duodenum and jejunum after overnight fasting. No striking differences were found in the total bacterial counts of the stomach when compared with children who were not malnourished. All 13 subjects had bacteria in the jejunum similar to range of non malnourished subjects i.e., 10^7 to 10^8 per ml. However, significant alterations were observed in the faecal flora consisting in: i) proliferation of facultative anaerobic bacteria more than that of strictly anaerobic bacteria, ii) frequent absence of *Bifidobacteria* and iii) colonization with *Proteus*, *Pseudomonas* and other Gram-negative bacilli which were not indigenous of faecal microflora. *Bacteroides* and *Veillonella* were not present in most of the subjects.

2.4.5.2 Diseases

Evaluation of the faecal flora in relation to disease occurrence was performed by Mata *et al.* among 12 infants during their 1st year of life. No changes were observed in faecal flora in relation to respiratory tract infections, uncomplicated measles, whooping cough, or in exanthematic diseases or skin infections. These results have been confirmed in six twins studied in the first 2 to 3 years of life. The similar scenario was observed in case of mild and moderate diarrhea in children. No alteration was noticed in faecal flora specifically in the total concentration of anaerobic and facultative anaerobic bacteria before the onset of the

disease, during disease and during convalescence. However, changes in some single component was observed e.g., i) decrease or absence of *Bifidobacteria* at the dilution tested (10^{-6}), ii) occasional proliferation of *Streptococci* or *Micrococci* to surpass the concentration of *Bifidobacteria* and iii) substitution of the common *Escherichia coli* for *Proteus*, *Providencia* or *Pseudomonas* (122).

Alterations in the composition of the gastrointestinal microflora frequently accompany acute diarrheal illness with dehydration. In most of the cases, the resident microflora may be eclipsed by an identifiable pathogen (40, 81). The striking change frequently observed is that, the proportion of anaerobes to aerobes is altered and aerobes predominate very often. *Bifidobacteria, Bacteroides* and *Veillonella* were found in lower numbers and conversely, *Enterobacteria* and *Staphylococci* were found in significantly greater numbers in children with diarrhea than their healthy counterpart (8). In patients with cholera, the concentration of Bacteroides in the faeces decreases to 10^5 cfu/ml, a reduction of 5-6 logarithms (82). Enterotoxigenic *E. coli* has been implicated as a major cause of diarrheal illness in young children in developing countries and in traveler's diarrhea. In a study of acute diarrhea in the tropics, Gorbach *et al* (81) isolated enterotoxigenic *E. coli* from stool and small intestine. Other coliform organisms, some of which produce enterotoxins, were also isolated from the gastrointestinal tract of some patients with "nonspecific" or viral diarrhea (40). In these patients, large numbers of coliforms are found in the small intestine, and faecal cultures often reveal high concentrations of *Klebsiella, Proteus, and Pseudomonas species* (9).

Alteration in faecal flora was also observed in shigellosis. In this disease, the anaerobic component decreased to levels comparable to those of the facultative flora or even lower. *Bifidobacteria* and *Bacteroides* were rarely found, whereas *E. coli* and other Entreobacteriaceae formed the bulk of the flora. Shigella were present in numbers as high or greater than those of the anaerobic component (122).

2.4.5.3 Antibiotics

In healthy state an ecological balance exists between the human host and microorganisms that colonize mucous surfaces and the skin. The individual indigenous microbiota is relatively stable at each ecological habitat, whereas there are greater differences between individuals depending on variations in diet and habits. The normal microflora represents a complex and important ecological system and alterations in the flora may give rise to serious clinical implications. Administration of antimicrobial agents causes disturbances in the ecological balance between host and microorganisms. To what extent disturbances occur depends on the spectrum of the agent, the dose, the route of administration, pharmacokinetic and pharmacodynamic properties and in-vivo inactivation of the agent (199).

Antibiotics of the penicillin group e.g., ampicillin, amoxicillin are broad-spectrum antibiotic and influence the intestinal microflora moderately and most often resulted in emergence of resistant *Enterobacteria*. In several studies, the authors studied the impact of orally administered ampicillin or amoxicillin on the normal intestinal microflora and observed that the numbers of *Enterococci, Streptococci, E. coli* strains, *Eubacteria* were suppressed and resistant strain of *Klebsiella, Enterobacter*, anaerobic Gram positive rods, *Bacteroides* and yeasts were increased significantly (26, 38, 64).

The spectra of cephalosporins are wider than that of penicillin and greater disturbances in the normal microflora was observed. Ceftriaxone, the mostly used antibiotic of this group has a profound negative impact on the total number of anaerobic bacteria of intestine (216). Another antibiotic clindamycin is mainly excreted in bile leading to very high concentration in faeces and causes great ecological disturbances. The effect of clindamycin on the intestinal flora of healthy subjects was investigated by Orrhage *et al.*, 1994 and an increase in *Enterococci* and *Enterobacteria* other than *E. coli* and a substantial decrease in total anaerobic bacteria were observed. The numbers of *Lactobacilli*, *Clostridia* and *Bacteroides* were decreased and *Bifidobacteria* disappeared under the detection limit. *C. difficille* was isolated in seven of the ten participants and of these three strains were toxin-positive (145).

In a study by Brismar et al., 1991, the effect of erythromycin on intestinal flora was investigated. The flora of the anaerobic population e.g., *Lactobacilli, Bifidobacteria,*

Clostridia and *Bacteroides* and aerobic *Streptococci* and *Enterobacteria* were strongly suppressed during the drug administration, but simultaneously resistant strain of *Enterobacteria*, *Staphylococci* and *Clostridia* was evolved (31).

The impact of vancomycin on the intestinal microflora was studied in connection with the evaluation of a probiotic product containing viable *Enterococcus faecium*. During oral administration, the total number of *Enterococci* decreased significantly and an overgrowth of *Klebsiella* species was observed. Among the anaerobic flora, the level of Lactobacilli was increased and the numbers of *Bifidobacteria*, *Clostridia*, and *Bacteroides* was decreased (110).

Oral administration of metronidazole to patients with respiratory tract, intra-abdominal or urinary-tract infections resulted in slight changes in the numbers of *Enterococci* and *Enterobacteria* in the faecal microflora. Only minor changes observed were in the anaerobic faecal microflora (140).

Wistrom *et al.*, 1992, examined the ecological effects on the intestinal flora of short term treatment with ciprofloxacin in people traveling to Mexico. A significant decrease of *Enterobacteria* and a smaller increase in the numbers of anaerobic cocci and *Bifidobacteria* was observed. *E. coli* and *Bacteroides* species with increased MICs of ciprofloxacin were isolated after treatment (218).

2.5 Functions of the human gut Microbiota

Although the gut does not secret enzymes capable to digest the residual food components, the colonic microbiota does that job in an excellent example of symbiosis. Collectively, the flora possess a metabolic activity that can be considered a virtual organ within an organ (28).

2.5.1 Fermentation of carbohydrates and proteins

Fermentation is an important component of the normal large bowel activity through which anaerobic bacteria break down dietary substrates and obtain energy for growth and the maintenance of cellular function. The major substrates for fermentation are dietary carbohydrates that have escaped digestion in the upper gastrointestinal tract. These include starch, Non-starch polysaccharide (NSP) and some simple sugars and alcohol.

Starch that enters the colon, also called resistant starch. Many factors may alter the digestive capacity of starch e.g., its origin, its processing as well as physiological and pathological conditions of the host. The origin of the starch is often considered an intrinsic factor of digestibility. Food particle size can affect starch digestion by amylase because smaller particles of larger surface area relative to volume are digested more rapidly than larger particles. In raw foods, starch is present as crystalline granules that can be surrounded by other plant materials that can inhibit access of amylase to them. Processing is considered an extrinsic factor of digestibility. Raw starches are highly resistant to enzymatic hydrolysis compared with cooked or gelatinized starches, while retrograded starch (stored after cooking) is less hydrolyzed than gelatinized starch (51). When starches are cooked with water, the granules become gelatinized to a degree that depends on temperature, proportion of water and time of cooking, but above 90°C much of the crystalline order is lost through breaking of the intermolecular forces allowing penetration of water (125). Among the host factor, pancreatic α-amylase concentration in the duodenal juice is low at birth and increases gradually with age. A congenital defect in amylase is very rare but secondary deficiency is frequently observed in severe malnutrition.

Normal native starches are composed of two types of polysaccharides: amylose (15-30%) and amylopectin (70-85%). Amylose is structurally a linear polymer of anhydroglucose units (α-1, 4 linkage), of molecular weight approximately between 40 and 340 kDa, the chains

containing 250 to 2000 anhydroglucose units. Amylopectin is considered to be composed of anhydroglucose chains (α-1, 4 linkage) with many branch points (α-1, 6 linkage); the molecular weight may reach as high as 80 000 kDa. Amylopectin consists of short chains of α-D-glucan (12-70 residues, average 20). Increasing the proportion of amylose in starch (rice, maize, barley) causes the resistant character of the starch to be increased to digestive amylases (214). Starch needs to be completely depolymerized to glucose before it can be absorbed in the small intestine. Starch-degrading enzymes are grouped into different families depending on their structure and include α- amylases that hydrolyse α-1,4- linkages, type I pullulanases that specifically cleave α-1,6- linkage and amylopullulanases that possess both α-1,4- and α-1,6- activities.

In the intestinal lumen, pancreatic α-amylase cannot hydrolyze the (1-6) branching links and has relative specificity for (1-4) links adjacent to these branching points. So, after digestion and absorption of starch in highest efficiency in small intestine there is chance to remain some undigested starch in very small amount. Some evidence from intubated humans indicated that free glucose and starch are found in the terminal ileum, which points to the passage of starch into the colon (68, 76). This undigested starch fraction has been called resistant starch. Englyst *et al.* (1992) classified resistant starches according to factors which are intrinsic to the food or to the processes used in its preparation (67). The classification is presented in Table 4.

Table 4. Classification of resistant starches.

Type of Starch	Example of occurrence	Probable digestion in small intestine
Rapidly digestible starch (RDS)	Freshly cooked starchy foods	Rapid
Slowly digestible starch (SDS)	Most raw cereals	Slow but complete
Resistant starch		
1. Physically indigestible starch	Partly milled grains and seeds	Resistant
2. Resistant starch granules	Raw potato, banana and High amylose maize starch	Resistant
3. Retrograded starch	Cooled and cooked potato, bread and cornflakes	Resistant
4. RS4[1]	Dextrins (chain linkage altered)	Resistant

1. A relatively new classification that refers to specific chemically modified or repolymerized starch.

Another important source of fermentable substrate to reach the colon is non starch polysaccharide (NSP), e.g. cellulose, hemicellulose, pectins, inulin, guar and plant gums. NSPs are also called dietary fiber and mainly found in the plant cell walls. Intestinal bacteria ferment 50% of cellulose and 80% of other fibres. Other carbohydrate sources available for fermentation are non-digestible oligosaccharides, various sugars and sugar alcohols e.g., lactulose, xylan, arabinogalactan, rhamnose, palatinose, sorbitol, xylitol, maltitol, lactitol (46). In addition, proteins and amino acids are very effective as growth substrates for colonic bacteria. These include mainly connective tissue protein e.g., elastin, collagen and serum albumin, as well as bacterial protein released from cell lysis. The intestinal mucosa is renewed every 2-3 days (10). Mucus degradation by colonic microflora has been well documented as well as the breakdown of mucopolysaccharide chondroitin sulfate. These are made up of glycoprotein and add a further 3-5 g for fermentation in the large intestine (43).

Total substrate availability in the human adult colon is 20-60g carbohydrate and 5-20g protein/day (43, 45). The short chain fatty acids (SCFAs) e.g., acetate, propionate and butyrate and gases H_2, and CO_2 are major products of the microbial fermentation of dietary carbohydrates in the human colon. The other significant end- products that are produced in

minor quantity from carbohydrate fermentation include lactate, ethanol, succinate, formate, valerate, caproate and CH_4. Gases produced from fermentations are eliminated both in breath and as flatus. Branched-chain fatty acids such as isobutyrate, 2-methyl- butyrate and isovalerate are formed from the fermentation of amino acids. The other end-products of protein breakdown include NH_3, phenols, indoles and amines, some of which have toxic properties (111). The pathways of carbohydrate and protein breakdown are presented in Figure 5 and 6 (170).

About 95-99% of SCFAs produced by bacterial fermentation are rapidly absorbed from the colonic lumen (181). At least 60% uptake is occurred by simple diffusion of protonated SCFA in a concentration-dependent manner (169), while the remainder occurs by cellular uptake of ionized SCFA involving co-transport of Na^+ or K^+ (74). SCFA uptake is associated with a transport of water and electrolytes from lumen to epithelial cells and thus acts as an antidiarrheal agent. Specifically acetate is metabolized in muscle, propionate in liver and butyrate in colonocytes to provide energy to respective tissues.

In the faeces, molar concentration for acetate is 60-80 M, propionate is 14-22 M and butyrate is 8-23 M (44). The amount of SCFA produced in human subjects is very

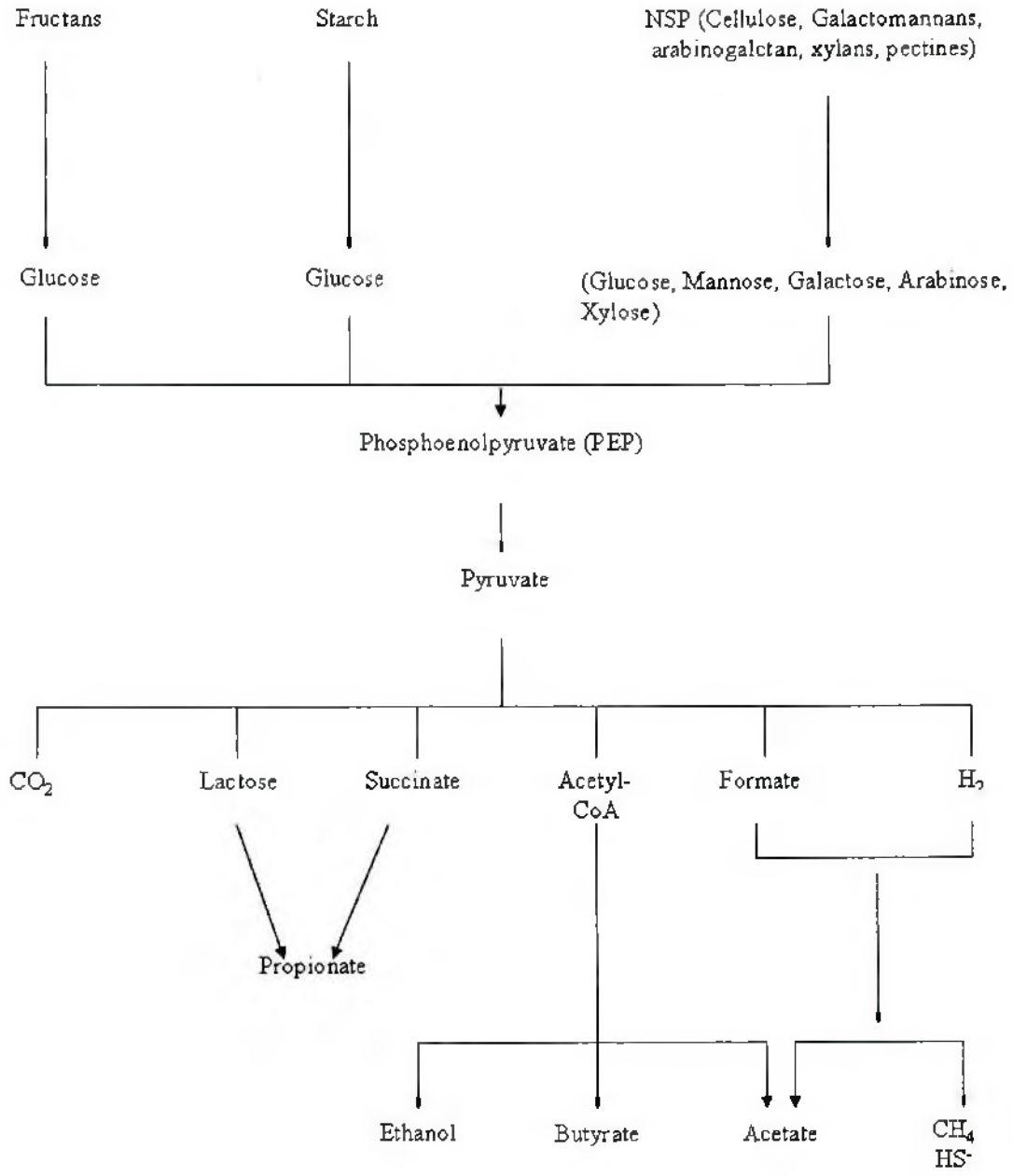


Figure 5. Pathways of polysaccharides breakdown in the gut microbiota and the principal end products.
(simplified from Macfarlane *et al.*, 2000).

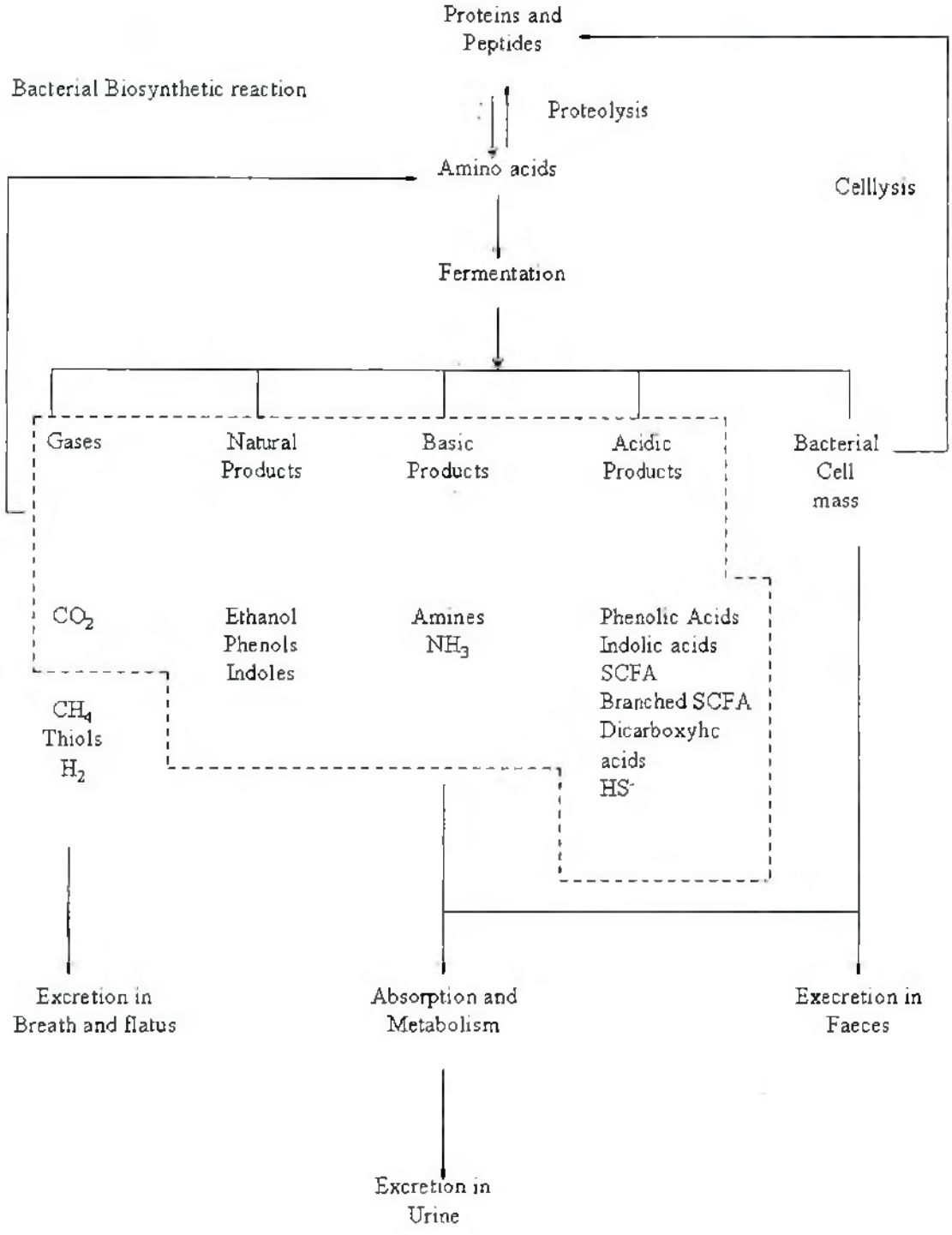


Figure 6. Mechanisms of protein breakdown by bacteria in the large intestine and the major end products.
(simplified from Macfarlane *et al.*, 2000).

difficult to determine by direct methods. Among the indirect methods studies of arterio-venous differences across the gut indicate that 300-500 mmol are produced each day. Another mostly used indirect method is to estimate the concentration of SCFAs excreted in faeces which range from 70 to 140 mM per L (171). Acetate is synthesized mainly by three pathways in the cell: i) Embden-Meyerhof-Parnas (EMP) pathway (47%), ii) Wood-Ljungdahl or CO_2 reduction pathway (27%) and by iii) Bifidobacterium pathway (21%). Propionate and butyrate are formed by CO_2 fixation pathways and classical routes of acetyl-S-coenzyme A condensation pathways, respectively (126). The general reaction of SCFA production and overall stoichiometry has been summarized for a hexose (73) as follows:

$59\ C_6H_{12}O_6 + 38\ H_2O \rightarrow 60\ CH_3COOH + 22\ CH_3CH_2COOH + 18\ CH_3CH_2CH_2COOH + 96\ CO_2 + 268\ H^+ + \text{heat}$ (205).

As a bi-product of fermentation, gut bacteria also synthesize and excrete vitamins in excess of their own needs which can be absorbed as nutrients by the host (171). Enteric bacteria secrete vitamin K and vitamin B12 and lactic acid bacteria synthesize certain B vitamins. Although this source of vitamins generally provides only a small part of the daily requirement, it makes a significant contribution when dietary vitamin intake is low.

2.5.2 Stimulation of immune system

Gut flora have a continuous and dynamic stimulation on the host's gut and systemic immune system. The bacteria are key in promoting the early development of the gut's mucosal immune system both in terms of physical components and function and continue to play a role later in life in its operation. In normal individuals contact of the ingested bacteria and the immune system occur only in the gut associated lymphoid tissue (GALT). The human intestine represents the largest mass of lymphoid tissue in the body, containing over 10^6 lymphocytes/g of tissue. In addition, about 60% of the total immunoglobulin produced daily is secreted into the gastrointestinal tract (171).

Intestinal immune cells are organized in different compartments: aggregated in follicles, Peyer's patches, lamina propria and distributed within the mucosa as diffuse lymphocyte populations; and in the epithelium (152). The Peyer's patches are more accessible to micro-

organisms than other epithelial surfaces of the gut, because they have reduced numbers of the mucus-secreting goblet cells. In addition, the epithelial layer of the Peyer's patches contains specialized transport cells called M-cells, which lack microvilli and are able to phagocytose both soluble antigens and micro-organisms. The lymphocytes activated within Peyer's patches disseminate via mesenteric lymph nodes, thoracic duct and the bloodstream back to the lamina propria, and traffic between other secretory tissues, including the respiratory tract and the lachrymal, salivary and mammary glands. Two IgA subclasses are available in gut e.g., IgAl and IgA2. IgAl immunocytes predominate in the small-intestinal mucosa, while IgA2 are most frequent in normal colonic mucosa. The digestive flora is the major antigenic stimulus responsible for the migratory pathway and maturation of precursor lymphoid cells present in the Peyer's patches. Consequently, it acts on the development and maturation of the IgA plasmocytes. In germ-free mice, IgA-plasmocyte number is decreased tenfold as compared with controls. In addition, Gram-negative bacteria such as *Escherichia coli* and *Bacteroides* play an important role in this immunologically non-specific effect.

As soon as an infant is born, bacteria begin colonizing its digestive tract. The first bacteria to settle in are able to stimulate the immune response, making them more favorable to their own survival and less favorable to competing species; thus the first bacteria to colonize the gut are important in determining the person's life long gut flora makeup (77, 184). Bacteria can influence the phenomenon known as oral tolerance in which the immune system is less sensitive to an antigen once it has been ingested. This tolerance mediated in part by the gastrointestinal immune system and in part by the liver, can reduce an over reactive immune response like those found in allergies and auto immune disease (102).

2.5.3 Protection against pathogens

Resident bacteria present a crucial line of resistance to colonization by exogenous bacteria and, in this way they are highly important in prevention of invasion of tissues by pathogens. This phenomenon is also called "barrier effect". In contrast to control animals, germ-free animals are highly susceptible to infection by exogenous microbes (15). Colonization resistance also maintains the restricted growth of opportunistic bacteria that are present in the gut. The equilibrium between species of resident bacteria provides stability in the microbial population within the same individual under normal conditions. However, use of antibiotics can disrupt the ecological balance and allow overgrowth of species with potential pathogenicity such as toxigenic *Clostridium difficile*, associated with pseudomembranous colitis (208).

Furthermore, bacteria compete for nutrient availability in ecological niches. The host actively provides a nutrient that the bacterium needs, and the bacterium actively indicates how much it needs to the host. This symbiotic relationship prevents unwanted overproduction of the nutrient, which would favor invasion of microbial competitors with pathogenic potential for the host. Finally, bacteria can inhibit the growth of their competitors by producing antimicrobial substances or peptides called bacteriocins (33, 109). The role of bacteriocins is mainly restricted to localized niches.

2.5.4 Production of toxic agents

Environmental factors such as diet have a major role in development of sporadic colon cancer. Dietary fat and high consumption of red meat, especially processed meat, are associated with high risk of this disease. By contrast, a high intake of fruits and vegetables, whole grain cereals, fish, and calcium has been associated with reduced risk of colon cancer (25). Intestinal microbiota plays a part in initiation of colon cancer through production of carcinogens, cocarcinogens, or procarcinogens. In healthy subjects, diets rich in fat and meat but poor in vegetables increase the faecal excretion of N-nitroso compounds, a group of genotoxic substances that are known initiators and promoters of colon cancer. Another group of carcinogens of dietary origin are the heterocyclic aromatic amines that are formed in meat

when it is cooked. Some intestinal microorganisms strongly increase damage to DNA in colon cells induced by heterocyclic amines, whereas other intestinal bacteria can uptake and detoxify such compounds. Sulphate reducing bacteria have an association with inflammatory conditions of the intestine. Bacteria that reduce sulphate are a heterogeneous group, but they have a common feature of activating sulphate to adenosine-5′-phosphosulphate (APS). Anaerobic bacteria then reduce this molecule to form bisulphite and then hydrogen sulphide. These two radicals are corrosive agent for the tissue (201).

2.6 Molecular ecology of gut microbiota

2.6.1 Techniques to study gut microflora

Until very recently gut microflora has been studied mostly by conventional culture techniques and these days microbiologists mostly prefer molecular biological tools for this purpose.

2.6.1.1 Conventional culture techniques

The analysis of the intestinal microflora has relied, almost completely, on the use of traditional bacteriological methods of culture, microscopy and biochemical properties of the bacteria (200). Although traditional cultivation methods are generally tedious, expensive, and are probably more open to misinterpretation with intestinal samples, they remain the "gold standard" for identification of bacteria. The growing of bacteria in laboratory culture media is of great importance in microbial ecology. Nevertheless, the predominant species of human intestinal microflora are non culturable because of their obligate anaerobic and extremely oxygen sensitive nature. Not only that, due to complex nutritional requirements and lack of beneficial effects that they receive from complex host-microbe and microbe-microbe interaction, recent estimates of culturability range from only 15 to 58% in the laboratory even when excellent bacteriological culture methods are used (197, 201).

The large discrepancy between the viable plate count and total direct microscopic count of bacteria is called the "great plate count anomaly", which described the huge bias due to cultivation (100). The example of this phenomenon is found not only in gut microbiota but also in water and soil ecosystem. Only 0.001 to 0.1% bacteria were cultivable among the total flora from a sea water sample (154). Again, total bacterial community in a forest soil is very high and reached 4000 different bacterial clones of standard genome size analysed by DNA reassociation and heterogeneity technique (206). Sometimes bacteria undergo "viable but non culturable" condition due to environmental stress e.g., freshwater, salt water or low temperature during which they can be enumerated microscopically but do not grow in plates. Examples are available for *Salmonella enteritidis*, *Vibrio cholerae*, *V. vulnificus* (101, 134, 167). But at the same time unknown species can also be noncultivable (12).

2.6.1.2 Molecular biological techniques

Microbial diversity within the human gut is currently grossly underestimated. However, the advent of molecular biology has revolutionized the identification of microorganisms. In particular the analysis of ribosomal RNA (rRNA) sequences provides an immensely powerful tool for determining the evolutionary interrelationships of micro-organisms. The high specificity and cumulative nature of rRNA sequence data have spurred the discovery and recognition of new biodiversity (27). 16S rDNA contains regions conserved across all bacterial species interspersed with regions (V1 to V9) in which the sequences are variable among bacterial types. Sometimes, the variable region sequences are highly species-specific. Comparison of 16S rDNA similarities can therefore be used in the identification of bacterial species and consequently, in the analysis of bacterial communities (200).

Some features of ribosomal RNA specifically of 16S rRNA are presented below which made it suitable for utilization as the molecule of molecular chronometer:

1. Ribosomal RNAs are universally distributed among all the bacteria.
2. It is functionally homologous in all the organisms (protein-synthesis).
3. Ancient molecule.
4. No lateral gene transfer of 16S rDNA has been observed (215)
5. The molecule contains highly conserved and variable regions. This mosaic property allows evaluating the distance and closeness between sequences.

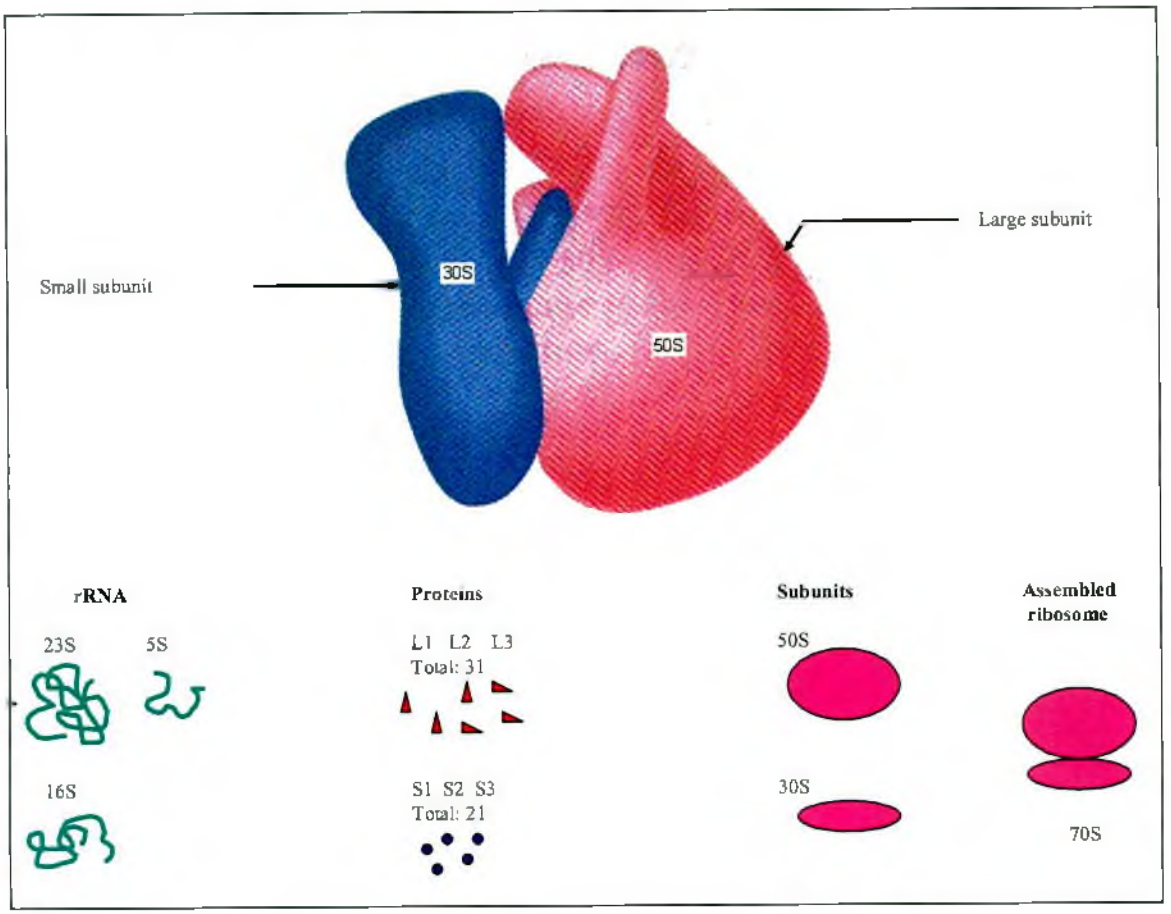


Figure 7. Structure of prokaryotic ribosome.

16S rRNA is present in the 30S subunit of prokaryotic ribosomes. The prokaryotic ribosomes are made of two subunits: 50S and 30S and their combined sedimentation rate is 70S. Though the ribosome is small in size their internal structure as well as function is not simple. The 50S unit is composed of two rRNA molecules: 23S (2904 nucleotides) and 5S (120 nucleotides) and 31 proteins. The 30S unit contain 16S rRNA (1542 nucleotides) and 21 proteins (Figure 7). The two subunits have different shapes. The 30S subunit exhibits a base, a platform and a head. The 50S subunit has two faces (back and front), a ridge, a central protuberance and a stalk. A tunnel is located inside the molecule. The weight of a ribosome is ~ 3 x 10^6 daltons and it measures 20 to 30 nm in diameter. In contrast to prokaryote ribosome, eukaryote ribosome possesses four rRNAs and the sedimentation rate is 80S. Irrespective of cellular type, ribosomes are actively functional for protein synthesis. They assemble mRNA, tRNA and amino acids to synthesize polypeptides.

The variable and conserved domains of 16S rRNA are highly informative for comparison and identification of bacteria. The conserved domains allow comparison of organisms that are very distant. While variable regions allow closely related organisms to be distinguished. The size of 5S rRNA is not sufficient for phylogenetic purposes and 23S is somewhat excessive long leading to increased cost for analysis. The 16S rRNA is long enough to be informative and statistically significant, so, it is routinely used to characterize bacteria.

2.6.2 A molecular view of the gut microbiota

Human large intestine is one of the most densely populated but less studied bacterial ecosystems in nature. This microbiota contains more bacterial cells than all of our body's other microbial communities together. Classification of these bacteria based on comparative analysis of 16S rRNA sequences into phylogenetic groups was commenced in 1980s when molecular taxonomic tools were developed (97). Designing probes and primers for all bacteria (universal) and for specific bacteria form 16S RNA and by applying molecular methods e.g., Dot Blot, Insitu hybridization, DGGE/TTGE, PCR and cloning of 16S rRNA, the diversity, quality and quantity of gut microbiota was assessed. It is now clear that majority of cultured bacteria from gut are assigned into three phylogenetic groups e.g., the *Bacteroides* group, the *Clostridium* group and the *Bifidobacterium* group (57, 108, 119, 217).

The *bacteroides* group is monophyletic and members of this group form the large bacterial phylum named *Cytophaga-Flavobacter-Bacteroides*, which represents one of the 12 bacterial phyla as defined by Woese, 1987 (219). Based on the diverse phenotypic characters, bacteria of this group was subdivided into three genera: Bacteroides, Prevotella and Porphyromonas (186). Their registration number in Ribosomal Database Project is 2.15.1.2. Several probes targeting the cluster of three genera or to the phylum has been designed and validated to detect members of the Bacteroides group in gut and natural environment (56, 119).

The bacteria of Clostridium group are divided into two subgroups e.g., the *C. coccoides* and *C. leptum* subgroup correspond to Clostridium cluster of XIVa and IV (41). Their registration number in RDP is 2.30.4.1 and 2.30.9.1.3 (118). A few bacterial genera are intermixed within each subgroup e.g., *Butyrivibrio, Coprococcus, Eubacterium, Lachnospira* and

Ruminococcus in *C. coccoides* and *Eubacterium, Ruminococcus, anaerofilum* and *Fusobacterium* in *C. leptum* group (118, 213).

Members of the *Bifidobacterium* group gathers in a monophyletic cluster called *Bifidobacterium* subgroup, RDP registration number 2.30.1.9.2.2. (118, 128). It has divided into five subgroup again e.g., *B. minimum*, *B. catenulatum*, *B. bifidum*, *B. lactis* and *B. infantis*. A genus-specific probe has been designed and validated by Langendijk *et al.*, 1995 to detect *Bifidobacteria* in faecal samples using in situ hybridization (108).

Chapter III

Aims and objectives

3. 0 Aims and objectives

Recent nutritional interventions have targeted colonic functions during rehydration of patients with infectious diarrhea and during recovery of children from malnutrition. Glucose-based oral rehydration salts (ORS) solution (G-ORS) is effective in rehydrating patients with acute infectious diarrhea. The basis for using standard G-ORS is that even during diarrhea glucose stimulates sodium and water absorption in the small intestine. Addition of complex carbohydrates would further stimulate water and electrolytes absorption in colon through the production of short-chain fatty acids. In proper designing of novel therapies that intend to use metabolism of intestinal microbiota requires a clear understanding of its composition and functions. However, study of colonic bacterial diversity during cholera is not well understood. In this perspective we have measured the quantity and biodiversity of colonic microbiota as a function of time in children treated with one of the three ORSs e.g., i) Glucose-ORS, ii) Glucose-ORS containing amylase resistant starch and iii) cooked rice ORS employing followings:

- A community fingerprint technique e.g., temporal temperature gradient gel electrophoresis (TTGE).

- In situ hybridization combined with flow cytometry using DNA probe targeted against 16S ribosomal RNA.

- High Performance Liquid Chromatography (HPLC) to study the concentration of short chain fatty acids produced in faeces of severely malnourished children with cholera.

All three parameters were checked in faeces of severely malnourished children without cholera and in healthy children for comparison.

Chapter IV

Materials & Methods

4.0 Materials and Methods

4.1 Study Site

The present study was conducted between April 2004 and March 2008 at Laboratory Sciences Division, Dhaka Hospital, ICDDR, B. I learnt the techniques in Department of Biology, CNAM, Paris.

4.2 Participants

The participants of this study was drawn from amongst the 175 malnourished children with cholera who were admitted under another clinical study entitled "Oral rehydration solution containing amylase resistant starch in severely malnourished children with watery diarrhea due to *V. cholerae* " at the Dhaka Hospital of ICDDR,B. This was a randomized, controlled clinical trial and the study enrolled malnourished male and female children between 6 month to 5 years with cholera. After 100 patients were enrolled in the parent study, patient inclusion was consecutively initiated in the

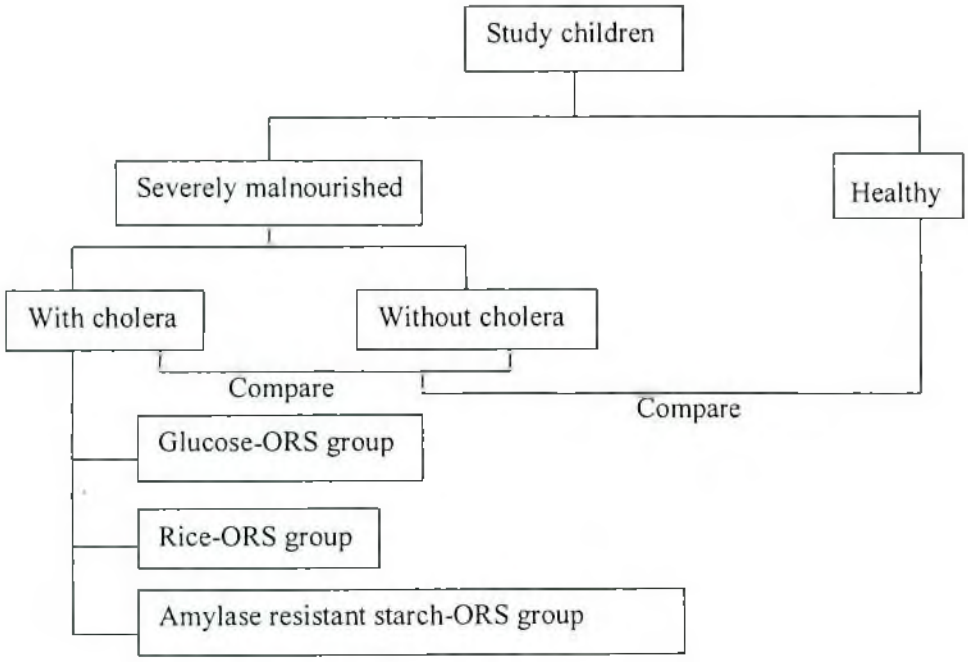


Figure 8. Flow diagram of study children.

present study, following the randomization of the parent study until 30 patients were recruited.

Inclusion criteria:
1. History of acute watery diarrhea of < 48 hrs.
2. Either gender.
3. Age 6 to 60 month.
4. Some or severe dehydration.
5. Wt for Ht ≤70% of the National Center for Health Statistics (NCHS) median with or without bipedal edema.
6. Dark field examination positive for *Vibrio cholerae*.
7. Those with other risk factors e.g., hypoglycaemia, hypothermia, hyponatremia, absence of breast feeding were eligible.

Exclusion criteria:
1. Bloody diarrhea.
2. Severe infection (e.g. severe pneumonia, clinical sepsis, Meningitis).
3. Those who received antibiotics / antimicrobial for the current illness.

The Research Review and the Ethical Review Committees of ICDDR, B approved the study protocol, and written, informed consent was obtained from the parents or guardians of each enrolled child. One of the investigators or a study physician obtained detailed medical history and performed thorough physical examination to confirm eligibility of each child before enrolling in the study. Eligible children were admitted to the Clinical Research Ward of the Dhaka Hospital of ICDDR, B and placed on a cholera cot. For comparison, age matched eleven severely malnourished children without cholera (weight for height ≤ 70%) from a nearby slum area in the Mohakhali area of Dhaka, close to the Dhaka Hospital, and six healthy children (weight for height ≥ 100%) of ICDDR, B staffs were also enrolled in the study. These children resided at their home, without any apparent illness, including diarrhea, and had no history of taking antibiotics or any drugs during the last two months (Figure 8).

4.3 Randomization

After confirming eligibility, children were randomly assigned to receive one of three formulations of ORS (Table 5) with the same salts composition but with different substrate composition namely glucose, glucose plus ARS, and rice powder (cooked). The ARS-based ORS (Hi-maize, Starch Australasia Limited, 170 Epping Road, Lane Cove NSW 2066, Australia) was of the same composition of the one used in an earlier study (158) that observed significant reduction in stool output in adults with cholera, and the rice-based ORS is the one routinely used at our hospitals (3). We modified the electrolyte composition of the ORS from that of ReSoMal, the WHO recommended formulation for management of diarrhea in severely malnourished children, containing potassium 40 mmol/L (to compensate for the deficit in malnourished children with diarrhea), and sodium 75 mmol/L (to compensate for stool losses in cholera) (92, 93). An experienced statistician of ICDDR, B, not involved in this study in any other way, prepared the randomization list, and prepared sequentially numbered sealed envelopes that contained inside a paper identifying the allocated ORS. The hospital pharmacist retained the list, and prepared the ORS in bottles marked with the patient's name and study (random) number (sequential number) when a new patient was enrolled.

Table 5. Compositions of three formulations of oral rehydration salt solutions (ORSs)

Ingredients	Glucose-ORS	Glucose + ARS-ORS	Rice-ORS
Glucose (mmol/L)	90	90	0
Rice powder (g/L)	0	0	50
Amylase-resistant starch (g/L)	0	50	0
Sodium (mmol/L)	75	75	75
Potassium (mmol/L)	40	40	40
Chloride (mmol/L)	87	87	87
Citrate (mmol/L)	10	10	10
Magnesium (mmol/L)	3	3	3
Zinc (µmol/L)	300	300	300
Copper (µmol/L)	45	45	45
Calculated Osmolarity -mosmol/L	305	305	215

Note: The glucose-ORS used is a modification of the WHO-recommended ReSoMal solution for malnourished children, containing higher amount of sodium (75mmol/L instead of 45 mmol/L) to address higher amount of stool sodium loss in cholera compared to other diarrheas (92). The amylase-resistant starch (ARS) used in the second group was identical to the one used in a clinical trial of adult patients with cholera (158). Rice-ORS was prepared by mixing the salt mixture and rice powder in 1050 ml of water and then boiled for 7 to 8 minutes, according to ICDDR, B's standard treatment.

4.4 Rehydration therapy

Children with severe dehydration were initially dehydrated using intravenous "Cholera saline", containing Na^+ 133, K^+ 13, Cl^- 98, and acetate$^-$ 48, all in mM, until their recovery from shock or severe dehydration. For those with some dehydration on admission or following initial intravenous rehydration, the estimated fluid deficit was corrected with one of the assigned ORSs in an amount of 100 ml/kg over a six-hour period. In addition, the same solution was used for matching ongoing stool losses in an amount of 5–10 ml/kg after each watery stool. Patients with high purging rates received ORS in amounts to match the ongoing losses. ORS therapy continued until the cessation of diarrhea. Children with some dehydration were randomized to receive the assigned ORS within an hour and those with severe dehydration within six hours of admission after intravenous rehydration.

4.5 Management of severe malnutrition

Following randomization, all patients were treated according to the protocol developed and followed for management of severely malnourished children at ICDDR,B hospital.(3, 92). Children without an apparent extra-intestinal infection received parenteral ampicillin in a dose of 100mg/kg plus gentamicin in a dose of 5 mg/kg, in four and two equally divided doses respectively, for five days. All patients received erythromycin for cholera in a dose of 12.5 mg/kg every 6 hours for 3 days. Those with oral candidiasis received nystatin oral suspension in a dose of 100,000 units every 6 hours until resolution of the condition. Beside this, they also received vitamin A, folic acid and multivitamin supplement. Breast-feeding was continued *ad libitum*, and supplementary feeding with a F100 diet (100 Kcal/100ml) was given in an amount of 10 ml/Kg (10 Kcal/Kg) for each feed, every two hours on the first day, and the amount was gradually increased to deliver 150 Kcal/Kg per day over the next 7 days according to the child's needs. In addition to the F100 diet, semi-solid food (cooked rice, lentils, and vegetables) was given to the older children during their convalescence and rehabilitation phase.

4.6 Measurements

Upon admission, the children were weighed and placed on a cholera cot, and a pediatric urine collector was applied for separate collection of urine from stools. Their body weight and weight of stools and supplemented foods were measured with an electronic scale (Sartorius, Germany) with a precision of 1g. All intakes i.e. ORS, plain water, intravenous fluids and foods, and outputs i.e. stool, urine and vomit were quantified for every 6 hour period of the acute phase of the study. Diarrhea was defined to have resolved, when the last watery stool was followed by two or more soft stools/formed stools or no stool for 12 hours. The duration of diarrhea was calculated in hours from the time of randomization to the last watery stool.

4.7 Specimen collection

Stool specimen was collected in universal container from each patient (10 patients in each ORS group) on day 0 (i.e. immediately after study enrollment and before administration of any treatment including rehydration solutions), on day 1 (24 hrs from randomization), and on days 2, 3, 7, 21, and 28. The patients were discharged from the hospital when they attained 80% weight for length and the follow up samples were collected from their homes. For comparison, faecal samples were also collected from eleven severely malnourished children without cholera and from six healthy children (Figure 9).

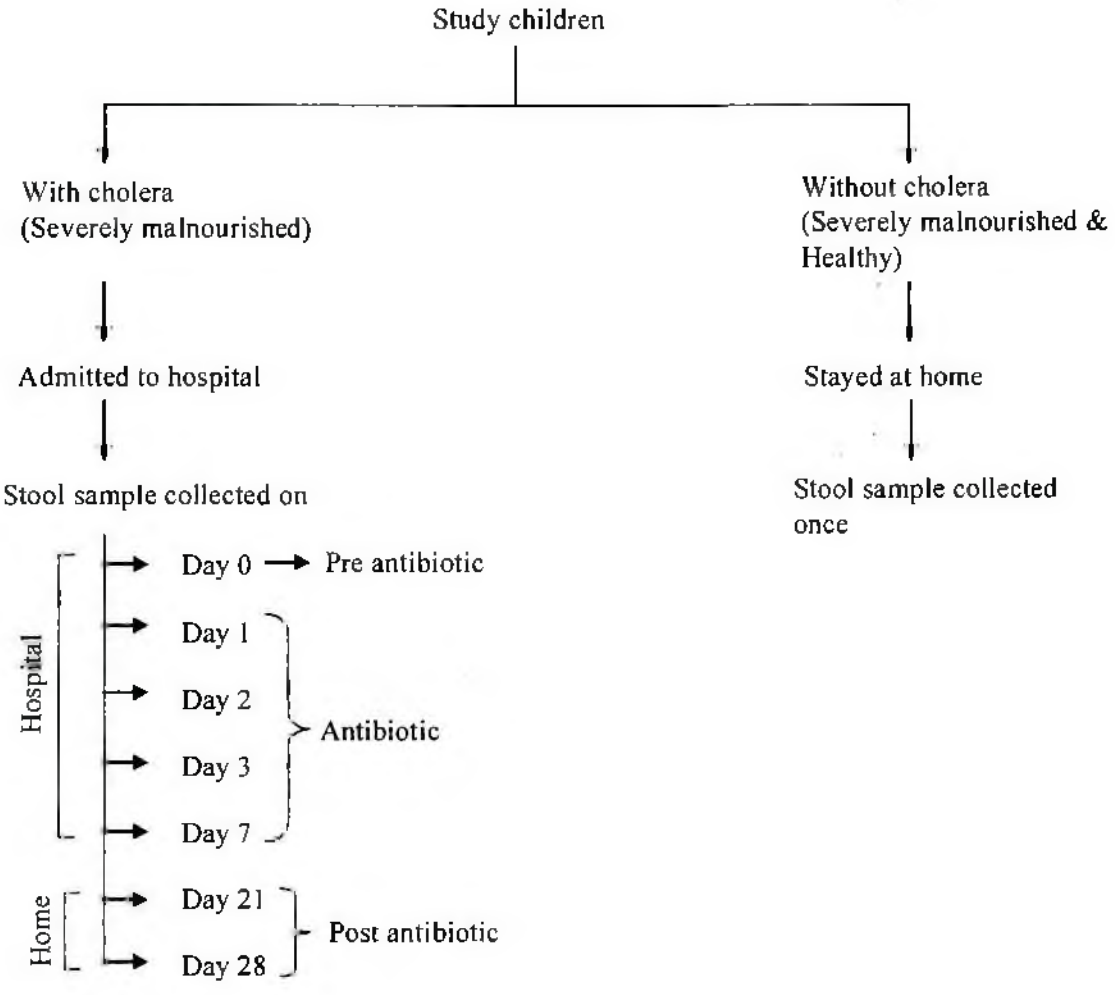


Figure 9. Planning of specimen collection.

4.8 Fixation of bacterial cell

After collection of specimen, the container was kept at 4°C for a maximum of 4 hours before processing. Faecal samples were fixed to stop the cellular metabolism, to inactivate lytic enzymes and endogenous RNases. However this step allowed conserving the morphology of bacterial cells and the integrity of the nucleic acids. 0.5 g (wet weight) of faecal sample were added to 4.5 ml of sterile phosphate buffered saline (PBS) and mixed for 3 to 5 minutes by vortexing with 5 - 6 glass beads (4 mm diameter) in a 15 ml screw capped falcon tube. The suspension was centrifuged for 10 minutes at 1000 rpm and one volume of the suspension was added to 3 volumes of 4% (wt/vol) paraformaldehyde (PFA) in PBS (130 mM NaCl, 3 mM NaH_2PO_4 $2H_2O$, 7 mM Na_2HPO_4 $12H_2O$, pH 7.2) for fixation. After overnight fixation at 4° C, the cells were harvested by centrifugation, 12000 rpm, 2 min, 4° C and washed twice with PBS solution to remove residual fixative, pelleted again and resuspended in cold PBS/ethanol (50:50), 4°C in a ratio of 1:1 and stored at –20° until further utilization for in situ hybridization (161).

4.9 Preservation of specimens

Faecal specimens were stored at -20°C until DNA extraction and short chain fatty acids extraction.

4.10 Biodiversity of faecal microbiota

4.10.1 Chromosomal DNA extraction

4.10.1.1 Reagents needed

The following reagents were used for chromosomal DNA extraction: Breaking buffer, Glass beads, Polyvinylpolypyrrolidone, Heating chamber, Phenol, 3M Sodium acetate (pH 5.2), 100% Isopropanol, Pure water.

4.10.1.2 Extraction procedure

One ml of watery stool was centrifuged for 10 minutes at 15,000 x g and the pellet was used for further processing. For semisolid and solid stools, 125mg (wet weight) was suspended in 625 µl of breaking buffer [0.8M guanidinium isothiocyanate, 4% N- lauroyl sarcosine, 20mM Tris (pH 8.0), 80mM sodium phosphate buffer (pH 8.0)] and incubated for Ih at 70°C. Afterwards, 750µl of glass beads 0.1mm in diameter (Sigma, St. Louis, MO, USA) and 15mg of PVPP (polyvinylpolypyrrolidone) were added. Bacterial cells were lysed in a vortex mixer at high speed (ten cycles consisting of 1 minute of vortexing and 1 minute of storage in ice). The mixture was centrifuged at 20,000 x g for 3 min at 4°C. After recovery of the supernatant, the pellet was washed three times with 200µl of TENP [50mm Tris-HCl (pH 8.0), 20mm EDTA (pH 8.0), 100mm NaCl and 1% (w/v) PVPP]. The four obtained supernatants were pooled. Nucleic acids were extracted with one volume of phenol. The aqueous phase was washed twice using chloroform-isoamylalcohol (24:1). Then one tenth of 3M sodium acetate (pH 5.2) and equal volume 100% isopropanol were added to aqueous phase and the eppendorf was kept at -20°C for 30 minutes. DNA was precipitated by centrifuging at 13,000 rpm for 20 minutes and the pellet was washed with 70% isopropanol, dried and resuspended in 50 to 100µl of sterile water and stored at -20°C. It was electrophoresed in 1% agarose gel (Figure 10), checked by UV transilluminator and documented by Kodac Gel Doc system, Japan.

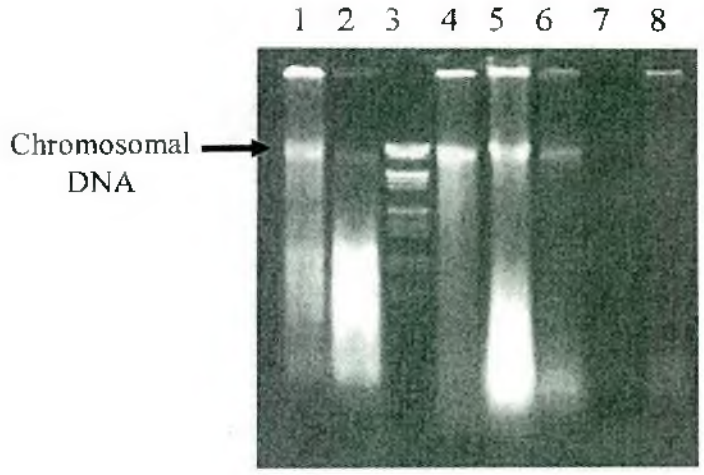


Figure 10. Faecal chromosomal DNA.
Lane 1, 2 and lane 4-8 showing DNA band. Lane 3, Marker VI of Boerhinger-Mannheim.

4.10.1.3 Quantitation of DNA

The quantity of DNA in each sample was measured by Gene Quant Pro, USA. 1 µl of DNA suspension was suspended in 99 µl of sterile deionized water in eppendorf tube and the optical density was measured at wavelength of 260 nm and 280 nm. The readings at 260 nm and 280 nm allowed calculations of the concentration of nucleic acids and proteins in the sample respectively because at these wavelengths the absorbance of nucleic acids and proteins is highest.

An optical density of 1 at 260nm corresponds to approximately 50 µg/ml double stranded DNA. The ratio between the readings at 260nm and 280nm provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have a value of 1.8 (O.D of 260nm/280nm). If there is any contamination in nucleic acids e.g., protein, phenol or salts the ratio of 260/280 will be significantly less than 1.8.

4.10.2 PCR amplification of 16S rDNA for TTGE

The polymerase chain reaction is a test tube system for DNA replication that allows a "target" DNA sequence to be selectively amplified several million-fold in just a few hours. This powerful technique was invented by Dr. Kary Mullis in northern California in 1983. This method uses a thermo stable DNA polymerase and two synthetic oligonucleotide primers that flank the DNA segment to be amplified. A three-step process referred to as a cycle is repeated upto 30-40 cycles consists of heat denaturation of the DNA, annealing of the primers and extension of the annealed primers with Taq DNA polymerase and dNTP. Two different primer sequences are used to bracket the target region to be amplified. One primer is complimentary to one DNA strand at the beginning of the target regions and second primer is complimentary to the other strand at the end of the target region and is so oriented that DNA synthesis occurs across the region between the primers. In addition, since the extension products are also complementary to, and capable of binding primers, each successive cycle essentially doubles the amounts of DNA synthesized in the previous cycles. This result in the exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles (175).

One PCR cycle consists of the following steps (Figure 11):

Step 1: Denaturation

During denaturation, the double stranded DNA is separated into two single strands by heating at 94° C. Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates are stronger covalent bonds, remain intact. This single stranded DNA then act as templates in the next round of DNA synthesis.

Step 2: Annealing-Primer binding to Target

The temperature is reduced to around 55° C to allow the primers to anneal to the target DNA.

Step 3: Extension

Once the primers anneal to the target DNA sequences, the temperature is raised to approximately 72°C and the Taq DNA polymerase begins the synthesis process at the region

marked by the primers. It synthesizes new double stranded DNA molecules, both identical to the original double stranded target DNA region. The new DNA strand then serve as a template for the enzymatic synthesis of new DNA strands in the next PCR cycle by the polymerase. Each PCR cycle theoretically doubles the amount of target DNA; one cycle yield 2, then 4, then 8, then 16, then 32 copies, resulting in exponential increases in the target which is sufficient to form a discrete band after 30-40 cycle when analyzed by agarose gel electrophoresis.

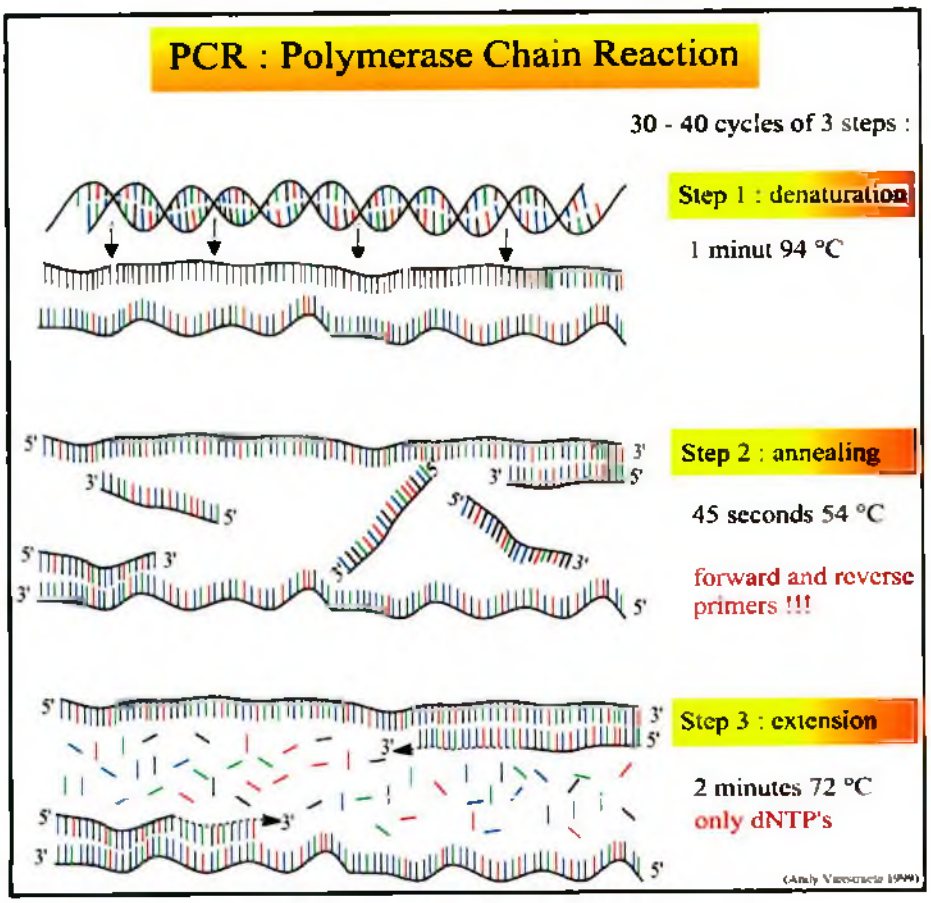


Figure 11. Different steps in PCR.

4.10.2.2 Components of reaction mixture

Preparation of reaction mix simply entails mixing of buffer, $MgCl_2$, dNTP, primer, Taq DNA polymerase and template. The out come PCR is influenced by the component of the reaction mixture.

$MgCl_2$

$MgCl_2$ at a particular concentration increases the activity of Taq DNA polymerase. However above a certain concentration, the activity of Taq DNA polymerase is inhibited by higher $MgCl_2$ concentration.

Deoxynucleotide tri-phosphate (dNTP)

dNTP concentration of about 200 µmole is enough to synthesize 12.5µg of DNA when half the dNTP are incorporated. dNTP chelate Mg^{2+} and thereby change the effective optimum magnesium concentration. Ultimately reduce the activity of *Taq* DNA polymerase.

Taq DNA polymerase

Taq DNA polymerase from extreme thermophile *Thermus aquaticus* is able to withstand the repeated heating and cooling steps of PCR and allow the synthesis of DNA at high temperature, which melt out the mismatched primer. Two forms of Taq DNA polymerase are now available the native enzyme purified from *Thermus aquaticus* a genetically engineered form of the enzyme synthesized in *E. coli* (AmpliTaqTm). Both forms of the polymerase carry a 5'-3' polymerization-dependent exonuclease activity, but they lack a 3'-5' exonuclease activity.

Template

For any PCR reaction plasmid DNA, chromosomal DNA or whole cell can be used as template. The two main concerns regarding to template are amount and purity of template. If the number of contaminants are present in DNA, it van decrease the efficiency of PCR. Again the amount of template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100ng of genomic DNA is sufficient to detect a PCR product from a single copy gene.

Primer concentration

A high primer concentration increases the probability of spurious priming and leads to the generation of nonspecific products. At the same time, it enhances the generation of primer dimmers. The concentration (C) of primer can be calculated by using the formula:

$$C\ (\text{pmole}/\mu\text{l or } \mu\text{M}) = \frac{\text{Abs. at 260nm} \times 100}{1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T}$$

Abs.= Absorbance at 260nm

n_A= number of adenine bases in primer

n_C= number of cytosine bases in primer

n_G= number of guanine bases in primer

n_T= number of thymine bases in primer

Cycling parameters

During each PCR cycle, the reaction mix is transferred between three temperatures. So cycling temperature should be chosen in such a way that it cause no mismatched hybrid or template primer hybridization. On the basis of GC content of primers and the length of the expected product, PCR programs are selected. Among the three steps of PCR, the annealing temperature is most important. If the temperature is too high than no hybridization takes place. If the temperature is too low then mismatched hybridization occurs. Usually the annealing temperature for a primer pair is generally calculated as 5°C lower or higher than the estimated melting temperature Tm.

4.10.2.3 Materials required for PCR reaction and Analysis

Instruments

- Thermal Cycler
- Pipettes with sterile tips box
- PCR work station or PCR Hood

Reagents

- Sterile deionized water
- 10X PCR buffer [500mM KCl, 200mM Tris-HCl (pH 8.4)]
- 10 mM dNTP (mixture of dATP, dGTP, dCTP, dATP)
- 25 mM $MgCl_2$
- 20 pmole primer 339 F
- 20 pmole primer 788 R
- Taq DNA polymerase
- Template DNA
- Agarose
- 0.5X TBE buffer
- Ethidium bromide

4.10.2.4 Procedure

Primer sequence

1. Eub339

   5′ CTC CTA CGG GAG GCA GCA GT 3′

2. Eub-788 (with GC clamp)

   5′ CCC CCC CCC CCC CGC CCC CCG CCC CCC GCC CCC GCC GCC C [GGA CTA CCA GGG TAT CTA A 3′

Specific PCR was carried out in 20μl volume and master mix was prepared according to the composition described in Table 6.

Table 6. Composition of PCR reaction mixture.

Components	Final concentration	Volume/ reaction (µl)
10X PCR buffer	1X	2.0
25 mM $MgCl_2$	2.5 mM	2.0
10 mM dNTP	0.8 mM	1.6
Primer 339 (20pmole)	0.4 pmole	0.4
Primer 788 (20pmole)	0.4 pmole	0.4
Taq polymerase	0.5 U	0.1
D/W H_2O	-	12.5
Template	100 ng	1.0

The PCR was then carried out in a thermal cycler 9700, Applied Biosystems USA, under following condition:

Initial denaturation at 94°C for 15min

at 96°C for 15 sec
at 55°C for 1min } 29cycles
at 72°C for 4min

Final extension at 72°C for 15min

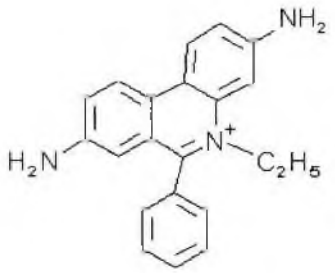
4.10.2.5 Study of the PCR product

When the PCR was completed the amplified product was studied by agarose gel electrophoresis to gain information about the DNA molecule that acted as the original template (Figure 12).

Agarose gel electrophoresis:

A portion of the amplified reaction mix (2 µl) was loaded in a 1% agarose gel and run at 100 V for 1h. The loaded DNA molecule was separated through the gel according to their molecular weight and charge. The low molecular weight DNA molecules come out from the gel first whereas the high molecular weight DNA comes later. A band representing the amplified DNA is visualized after staining the gel by ethidium bromide.

Preparation of gel: 1g of agarose powder were dissolved in 100ml of 0.5x TBE (Tris Borate and EDTA buffer) buffer and ethidium bromide (0.1 ng ml^{-1}) was added in gel to visualize the product.



Ethidium bromide formula

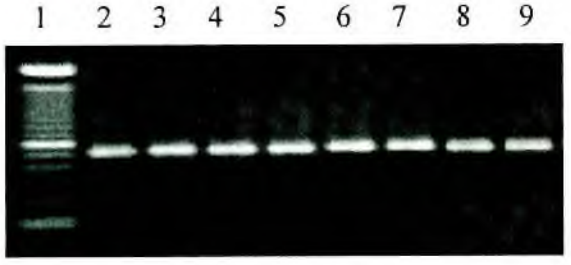


Figure 12. PCR product of 16S rDNA from faecal mixed chromosomal DNA by universal primer.
Lane 1, Marker 100 bp ladder. The amplicon size is 449 bp.

4.10.3 Temporal Temperature Gradient Gel Electrophoresis (TTGE) of 16S rDNA PCR products

The temporal temperature gradient gel electrophoresis is a powerful new technique that allows separating the different DNA sequences on the basis of their GC base composition. It uses the modified principle of DGGE (denaturing gradient gel electrophoresis) which require a chemical denaturing gradient. The separation techniques of DGGE were first described by Fischer and Lerman in 1983. The DNA fragment which is loaded in a polyacrylamide gel is obtained first through PCR and then migrates from negative to positive pole. During TTGE,

the gel contains constant concentration of urea and during electrophoresis, the temperature is increased gradually and uniformly (Figure 13). The result is a linear temperature gradient over the length of the electrophoresis run. Thus, a denaturing environment is formed by the constant concentration of urea in the gel in combination with the temporal temperature gradient. During migration through this gel, DNA fragments (double strand) undergo different degree of denaturation which depends on percentage and repartition of GC in the sequence. Denaturing of a DNA fragment reduces its mobility in the polyacrylamide gel and they stop their migration at the moment, they are completely denatured. Molecules with different sequences will stop migration at different positions in the gel. In practice, nearly all single base substitutions in PCR amplicons up to 500 bp could be detected by TTGE as well as by DGGE. A sequence of guanines (G) and cytosines (C) called GC-clamp (~ 40 nucleotides) is added to the 5'-end of one of the PCR primers and co-amplified with the target DNA. The GC clamp acts as a high melting domain and prevents the two strands of DNA fragment to be completely separated during denaturation. So, DNA sequences of different composition e.g., wild and mutant type DNA, 16S rDNA of different bacteria from a complex community e.g., soil, water, air, gingival and gut can be separated by these techniques.

Instrument

- The DCode™ Universal Mutation Detection System, Bio-Rad, USA.
- Magnetic Stirrer

Reagents

- Urea
- Tris-Acetate-EDTA (TAE) buffer, 50 X
- Acrylamide-bis acrylamide (37.5:1)
- Ammonium per sulfate
- TEMED
- SYBR® green I solution for nucleic acid staining (Sigma, St. Louis, MO, USA)

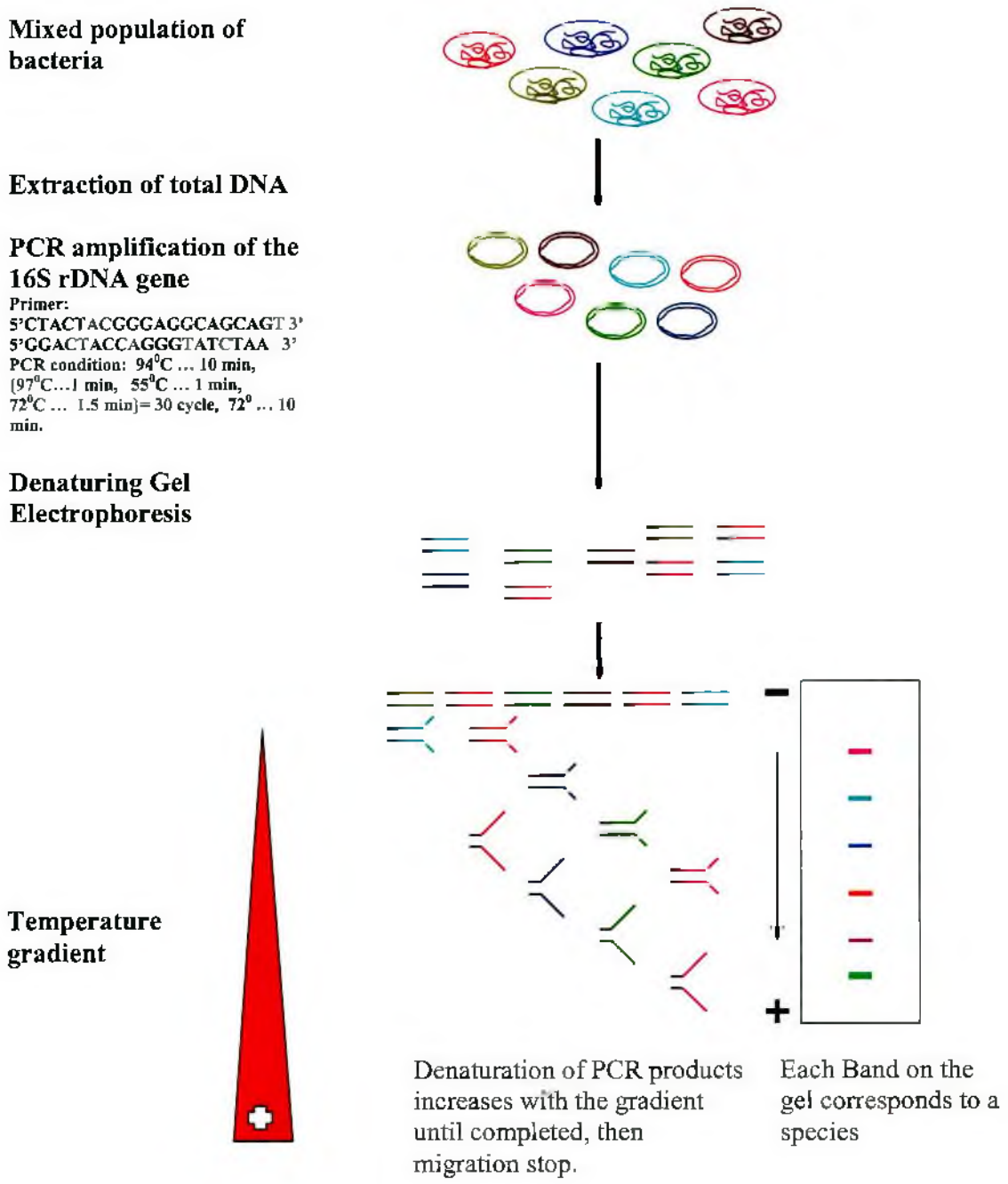


Figure 13. Principle of Temporal Temperature gradient Gel Electrophoresis (TTGE).

4.10.3.1 Assembling of gel casting apparatus:

A large plate, two spacers and a short plate were assembled together. Each sandwich clamp was placed on a side of assembled plates (locating arrows facing up and toward the plates). The screws were tighten enough to hold the plates in place. The sandwich assembly was placed in the alignment slot of the casting stand (short plate forward). The clamps were loosened, an alignment card was inserted to keep the spacers parallel to the clamps and plates and spacers were aligned. The screws were tightened until it is finger-tight. The gray sponge was placed onto the slot for each sandwich and it was placed on it facing the short plate forward. Press down the sandwich and turn the handles of the camshaft down. The comb was inserted (teeth at a slight angle).

4.10.3.2 Electrophoresis:

55 µL of TEMED and 550 µL of APS (10%) were added to 60 mL of acrylamide gel solution, homogenized gently and was poured with a syringe till the solution covers the combs. The combs were straightened and bubbles were checked. The gel was allowed to polymerize for 2 h. By this time, 7 L of 1.25 X TAE buffer was prepared from the 50X stock and the gel tank was filled. It was placed on a magnetic stirrer. Power, pump and heater switch were turned on: Temperature was set at 66°C and ramp rate at 200°C/h.

Once the gel was polymerized, the combs were removed, the wells were rinsed by 1.25X TAE buffer using a syringe. The entire front of the core gaskets was lubricated with water and the sandwich of glass plates were fitted on it. The core was turned and the second sandwich was attached. The upper buffer chamber was filled with 350 ml of running buffer, the seal integrity was checked and each well was cleaned again. The D-code was turned off and I waited for at least one minute. The temperature control module was removed on the lid stand and the core with glassplate sandwiches were placed in the buffer chamber. The temperature control module was replaced, the power, pump and heater were turned on and allowed to reach the temperature of 66°C.

The temperature control module was removed on the lid stand when the temperature was attained. 12 µl sample (PCR product) was loaded in each well (right to left for the front gel and left to right for the other). The temperature control module was replaced and all switches were turned on. When the temperature reached 66°C a pre-run was followed by the main electrophoresis were performed. Pre-run for 15 min, V=20V, A~18mA.

The temperature of the gel system was programmed to increase by 0.2°C per hour from 66°C to 70°C. Overnight electrophoresis at constant voltage, V=65V, A~67mA, T → 70°C, rr = 0.2°C/h. The magnetic stirrer was also turned on for proper mixing of the buffer and uniform distribution of the temperature.

Additionally, similarly obtained PCR products from known bacterial strains were loaded to allow standardization of band migration and gel curvature among different gels. This ladder consisted of DNA of the following organisms listed in migration order: *Prevotella* sp., *Vibrio cholerae, Enterococcus faecium, Escherichia coli, and Bifidobacterium longum.*

On the next morning when the temperature has reached 70°C (around 18h), the D-code has turned off. The core was removed from the tank and the gels were taken out. Gel staining was done in 1.25X TAE containing SYBR® green I solution (50 µl for 500 ml buffer) for 15 minutes. The gel was washed in 1.25 X TAE for 10 minutes and observed under UV and scanned using Gel Doc 2000 (Bio-Rad Inc., Hercules, CA, USA).

4.10.3.3 Analysis of TTGE profile

Temporal temperature gradient gel patterns were analysed using Diversity Database 2.1, part of the Discovery Series (Bio-Rad). The analysis is described in detail elsewhere (114) and is based on the method of Deplancke *et al.* who developed the original strategy (50).

In brief, bands were detected using the option "Detect Band" in defined lanes based on parameters selected. Before band detection, the background was subtracted from the lanes using the "Lane Background" option. Lane based background subtraction uses a "rolling disk" method of subtraction. A disk radius that is too large is resulting in poor removal of background and a disk radius that is too small is subtracting actual data. So, the disk radius

was adjusted as follows: by clicking on a lane, the lane profile was displayed and the disk radius was set empirically so that it touches actual points along the profile trace and subtracts actual background. Band detection was done by adjusting the parameters in "Detect Band" dialog box e.g., sensitivity, lane width, minimum density, noise filter etc., so that all the clear bands can be detected. .

Each band was defined by its relative intensity and relative front (Rf). The relative intensity is the intensity of a particular band in a lane expressed as a percentage of the total intensity data in the lane. Rf is the distance from the top to the band of a defined lane. In order to compare several gels, a normalized Rf derived from Rf was used to classify bands. This normalized Rf was based on the migration of a marker. A band set was stored containing classified bands defined as unique band types according to their normalized Rf.

Comparisons of TTGE profiles were performed using Dice's similarity coefficient (D_{sc}) analysis based entirely on the results of band classification. D_{sc} values were compared, based on the presence or absence of bands. Dice's coefficient is defined as follows:

D_{sc} = [2j/(a+b)] x 100 where j is the number of common bands between samples A and B; a and b are the total number of bands in samples A and B, respectively. This coefficient ranges from 0 (no common bands) to 1 (identical band patterns). Consequently, the distance between two TTGE profiles was: Distance = 1- D_{sc}

4.10.4 Cloning of *V. cholerae* 16S rDNA

V. cholerae O1 and O139 16S rDNA was cloned to utilize these bacteria as a marker in TTGE gel along with other known bacteria.

4.10.4.1 PCR amplification of the target DNA

1. Primer Eub339
   5' CTC CTA CGG GAG GCA GCA GT 3'

2. Primer Eub 1385
   5' GCG GTG TGT ACA AGR CCC
   R =A/G

Table 7. PCR reaction for cloning.

Components	Final concentration	Volume/ reaction (µl)
10X PCR buffer	1X	5.0
25 mM $MgCl_2$	2.5 mM	5.0
10 mM dNTP	0.8 mM	4.0
Primer 339 (20pmole)	0.4 pmole	1.0
Primer1385 (20pmole)	0.4 pmole	1.0
Taq polymerase	1.25 U	0.25
D/W H_2O	-	32.75
Template	100 ng	1.0
		50.0 µl

The PCR was then carried out in a thermal cycler 9700, Applied Biosystems USA, under following condition:

Initial denaturation	at 94°C for 10min	
	at 97°C for 1 min	25 cycles
	at 59°C for 1min	
	at 72°C for 1.5min	
Final extension	at 72°C for 15 min	

The amplicon size was 1046 bp.

4.10.4.2 Purification of PCR products

PCR products were purified and concentrated by QIAquick PCR purification Kit from QIAGEN. It contains a silica-gel-membrane for binding of DNA in high-salt buffer and eluting with low-salt buffer or water. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from DNA samples.

Procedure:

- Three PCR products of each serotype of *V. cholerae* were pooled (150 µl).
- 5 volumes of buffer PB was added to the PCR product and mixed.
- A labeled QIAquick Spin Column was place in a provided 2ml collection tube and the DNA with PB was applied in it. Centrifuged for 1 min at 13000 rpm.
- The flow-through was discarded and the QIAquick Column was placed back into the same collection tube.
- 0.75 ml buffer PE was added to wash the QIAquick Column and spinned for 1 minute. The flow-through was discarded and the QIAquick Column placed back into the same collection tube.
- Again another spin was done.
- The QIAquick Column was placed in a fresh clean 1.5 ml microfuge tube.
- 40 µl Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane to elute DNA and centrifuged. The eluent was taken in a new eppendorf tube and kept at -20° C.

4.10.4.3 Ligation of insert (PCR product) to vector

The reagents for ligation were thawed by placing on ice and briefly centrifuged.

Table 8. Ligation mixture

Components	Volume per reaction
Ligation master mix 2x	5 µl
PCR products	3 µl
p^{GEM} T easy vector	1 µl
T4 DNA ligase	1 µl
Total	10 µl

The components were carefully mixed and kept at 4° C for overnight. On the next morning it was used for transformation.

4.10.4.4 Transformation

Transformation is the last step in cloning procedure in which the vector containing the target DNA was transferred into a competent bacterial cell.

- A frozen tube with competent cells (*E. coli* DH10B, 50 µl) was placed on ice until thawed.
- 2 µl of ligation mixture was added, mixed gently and kept on ice for 30 minutes.
- The cells were heat shocked at exactly 42° C for 90 seconds and immediately kept in ice for 2 minutes.
- 950 µl SOC medium was added at room temperature and was kept at 37° C for 45 minutes.
- 100 µl SOC was spread on Luria agar plate containing ampicillin$_{100\ \mu g/ml}$, X-Gal $_{80\ \mu g/ml}$ and IPTG $_{0.5\ mM}$.
- The plates were incubated for overnight and on the following day the transformants were detected by blue / white screening. The white colonies were positive transformants.
- Ten colonies were picked up, numbered and subcultured.

4.10.4.5 Identification of clones by PCR

The clones were identified by PCR using primers targeted against the plasmid of the Transformant (Figure 14). The primers were T7 and SP6.

1. T 7 → 5' TAA TAC GAC TCA CTA TAG GGC GA 3'

2. SP 6 → 5' ATT TAG GTG ACA CTA TAG AAT AC 3'

Table 9. PCR reaction to identify clones of *V. cholerae*.

Components	Final concentration	Volume/ reaction (µl)
10X PCR buffer	1X	2.0
25 mM $MgCl_2$	2.5 mM	2.0
10 mM dNTP	0.8 mM	1.6
Primer 339 (20pmole)	0.4 pmole	0.4
Primer1385 (20pmole)	0.4 pmole	0.4
Taq polymerase	0.5 U	0.1
D/W H_2O	-	13.5
Template	100 ng	1.0
		20.0 µl

The PCR was then carried out in a thermal cycler 9700, Applied Biosystems USA, under following condition:

Initial denaturation at 94°C for 10min

at 97°C for 1 min
at 48°C for 1min } 35 cycles
at 72°C for 1.5min

Final extension at 72°C for 15 min

The amplicon size was 1233 bp.

4.10.4.6 Plasmid DNA extraction from the selected clones

The overnight culture of the clones in Luria-Bertani broth (5 ml) containing ampicillin was used for plasmid DNA extraction. 1.5 ml broth was centrifuged and the supernatant was discarded.

- the pellet was suspended in 250 µl of Buffer P1 (RNase was added).

- 250 µl of Buffer P2 was added and mixed thoroughly for 5 minutes by inverting the tube 4–6 times. Mixing was done gently and vortex was avoided to prevent the shearing of genomic DNA.
- 350 µl of buffer N3 was added and mixed immediately and thoroughly by 4-6 times inverting the tube. The solution was cloudy.
- The suspension was centrifuged for 10 min at 13000 rpm in a Table-top microcentrifuge.
- The supernatants were applied to the QIAprep spin column by decanting or pipetting and centrifuged for 1 min. the supernatant was discarded.
- The QIAprep spin column was washed by adding 0.75 ml buffer PE and centrifuged for 1 min.
- The supernatant was discarded and again centrifuged for 1 min to remove any residual wash buffer.
- The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 50 µl of buffer EB (10 mM Tris·Cl, pH 8.5) to the center of each QIAprep spin column, then waited for 1 min and centrifuged for 1 min.

The plasmid DNA was checked by agarose gel electrophoresis and by PCR using primer EuB-339 and EuB-788 (without GC clamp). Finally, the PCR was done by universal primer with GC-clamp and the product was run in TTGE (Figure 15).

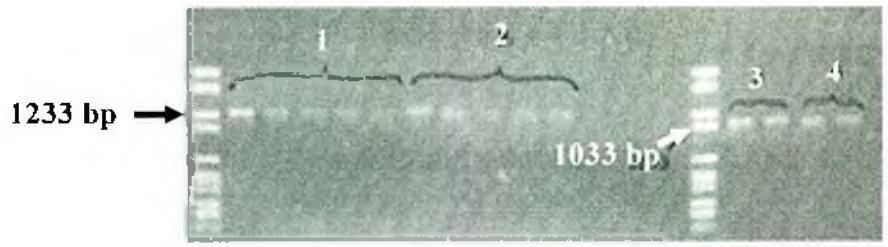


Figure 14. PCR product after transformation.
1 & 2 , PCR directly from transformant bacteria by primer T7 and SP 6. 3 & 4, PCR from extracted plasmid by Eub 339 & Eub 788 primer.

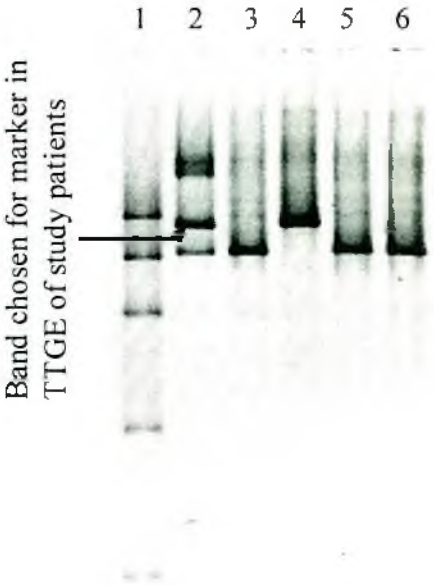


Figure 15. TTGE gel image showing *V. cholerae* 16S rDNA from parent and clones (colour inverted).
Lane 1, Marker; Lane 2, *V. cholerae* O1; Lane 3-6, *V. cholerae* clones. Clone in lane 4 was used for marker in TTGE gel.

4.10.5 Sequencing of TTGE bands

4.10.5.1 Principle of sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. In 1977, twenty-four years after the discovery of the structure of DNA, two separate methods for sequencing DNA were developed: the chain termination method (Sanger method) and the chemical degradation (Maxam Gilbert) method which used autoradiography to detect the location of the nucleotides. But now a days different genetic analyzer have been discovered which detect the nucleotides from fluorescence labeled DNA by laser beam instead of using X-Ray machine. Due to some difficulties with chemical degradation method, now-a-days chain termination method is mostly used. The art of this technique is known as Sanger sequencing after its brilliant pioneer, Frederic Sanger. This technique utilizes 2', 3'-dideoxynucleotide triphospates (ddNTPs), molecules that differ from deoxynucleotides by the having a hydrogen atom attached to the 3' carbon rather than an OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide. This technique involves the separation of fluorescent labeled DNA fragments according to their length on a polyacrylamide gel (PAGE). The base at the end of each fragment can then be visualized and identified by the dye with which it reacts. The time and labor intensive nature of gel preparation and running, as well as the large amounts of sample required, increase the time and costs of genomic sequencing. To alleviate these problems and due to improvement of technology now a days sequencing is done by automatic machine following the principle of Sanger's method.

4.10.5.2 Automated sequencing

In automated DNA sequencing a cycle sequencing kit is used (e.g. Big Dye™ terminator cycle sequencing ready reaction kits), that contains AmpliTaq DNA polymerase and both dNTP and dye labeled ddNTPs, and uses dye terminator cycle sequencing strategies. In this method only one strand of the desired DNA are copied with one primer and results in the linear increase of the number of copies of one strand of the gene. The growing chains are terminated when dye labeled terminators (ddATP, ddGTP, ddCTP, ddTTP) are incorporated. After cycle sequences, unincorporated chain terminators are removed from the reaction mix

with alcohol precipitation and the DNA fragments of different length, which ended on a fluorescent-labeled ddNTPs, are separated according to their length through capillary electrophoresis. Finally the nucleotide bases are detected in ABI Prism-310 Genetic Analyzer machine by the laser detection system.

There are four major preparatory steps for DNA sequencing

(1) Preparation of target DNA

(2) Cycle Sequencing

(3) Purification of the cycle sequence product

(4) Sequencing

4.10.5.3 Preparation of target DNA

Several bands were cut aseptically from patients TTGE gel which were parallel to the different bands in marker lane and 11 among them were sequenced. Gel cut bands were taken in microcentrifuge tube, 50 µl of PCR grade water was added in each tube and kept for overnight at 4°C. The target DNA was prepared from this water by PCR as described in section 4.10.2.4. Both the PCR e.g., PCR-TTGE and PCR-Sequencing were similar, the only difference was that, the primers used were without GC-clamp. The PCR product was purified by using commercially available column (MicroconYM-100). Briefly, PCR product was poured in a microcentrifuge tube-containing sieve. The tube was centrifuged at 5000 x g for 12 minute. Then the sieve was removed from the previous tube and placed on a new tube as upside down. Then again centrifuged at 10000 x g for 3 minutes. The sieve was discarded and the filtrate was used as purified template for sequencing. Then the DNA was quantitated in Gene Quant pro as described in section 4.10.1.3.

4.10.5.4 Cycle sequencing

In cycle sequencing the ABI–prism Big Dye terminator cycle sequencing ready reaction kits was used which contain all the required components for the sequencing reaction (dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, magnesium chloride and buffer). In this step only one strand of the desired DNA is amplified and the DNA fragments of different length, which has been terminated with different ddNTPs, were produced.

Table 10. Template size relative to quantity.

Template	Quantity
PCR Product	
100-200bp	1-3 ng
200-500bp	3-10
500-1000bp	5-20ng
1000-2000bp	10-40ng
>2000bp	20-50 ng
Single stranded	25-50 ng
Double stranded	150-300 ng
Cosmid Bac.	0.5-1 µg
Bacterial genomic DNA	2-3 µg

Table 11. Reaction mixtures of cycle sequencing

Reagent	Quantity
Terminator Ready Reaction Mix	4.0µl
Template	Quantity in the Table
Primer	3.2 pmol
Deionized water	to make 20 µl
Total volume	20µl

Programme of cycle sequencing:

96°C	1 min	
96°C	10sec	25 cycles
50°C	5sec	
60°C	4 min	
60°C	1min	

4.10.5.5 Purification of the Extension Products:

2.0 µl of 3M sodium acetate (pH 4.6) and 50.0 µl of 95% alcohol were added in each PCR reaction tube. It was mixed thoroughly and kept at room temperature for at least 15 minutes to precipitate the PCR products. It was centrifuged for 20 min at 13,000 rpm and the supernatant was aspirated. The pellet was washed with 250µl of 70% alcohol, by washing the wall of the tubes. Again centrifuged for 10 min and the washing steps were repeated twice. The pellet was dried by keeping the lid of the PCR tube and covered by perforated para film over the tube.

4.10.4.6 Sequencing of PCR product

Dried pellet was dissolved in 12-15µl of template suppression buffer and denatured for 2min in the PCR machine and immediately kept in ice. The product was transferred in the septa tube and placed on a 48- well tray. The tray was loaded on an ABI PRISM 310 automated sequencer (PE Applied Biosystems, USA) and samples injected in to a 47 cmx50 capillary coated with POP-6 (Performance Optimized polymer-6, PE Applied Biosystems, USA) polymer using electrokinetic injection at 320 volts/cm (15 KV for the 47 cm capillary, current at this voltage 5-8 µA) and electrophoresed using the Seq POP RAPID E run module with the DT POP6 mobility file. The sample fluorescent spectra during electrophoresis was processed into base sequence data using the ABI PRISM 310 Data Collection Software and the raw sequencing data was analyzed using the ABI PRISM DNA Sequencing Analysis Software (PE Applied Biosystems, USA). The sequences were matched with existing sequences of

GenBank by using BLAST (the Basic Local Alignment Search Tool, Version 2.0) of the national Centre for Biological Information (NCBI, http//www.ncbi.nlm.nih.gov).

4.11 Extraction and analysis of total short chain fatty acids (SCFAs) by High Performance Liquid Chromatography (HPLC)

Chromatography was first developed by the Russian botanist Mikhail Tswett in 1903 when he separated a colorful plant pigments through a column of calcium carbonate. Chromatography has since developed into an invaluable laboratory tool for the separation and identification of compounds, ranging from inorganic ions to complex biopolymers. In broad sense, chromatography is of two types: gas chromatography and liquid chromatography. In gas chromatography the sample is vaporized and passed through a mobile phase that is usually an inert gas e.g., helium or nitrogen through the stationary phase made of a microscopic layer of liquid or polymer on an inert solid support, inside a glass or metal tubing, called column. Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid carrying the sample through a column that is packed with irregularly or spherically shaped particles called stationary phase. It was discovered that better separation of the components of the mixture occurs if the particles in the stationary phase are very small. However, it was also found that if very small particles were used in the column, then the liquid passed very slowly through the column. Therefore, a pump is used to force the liquid through the column. Due to better performance is obtained when applying high pressure, the technique is called high performance liquid chromatography, HPLC (58).

HPLC was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. Modern HPLC has many applications including separation, identification, purification, and quantification of various compounds. New techniques e.g., autoinjecter instead of manual, fluorescence detection instead of UV detection, dual lamp, data integrated by computer and analysis, development of the micro-columns, and other specialized columns added to the convenient use of HPLC in many sectors. Now a days, it is used not only by biotechnological, biomedical, biochemical research and by the pharmaceutical industries, but also a variety of other fields including cosmetics, energy, food, and environmental industries are currently using HPLC (196).

A flow diagram of acids extraction is given below:

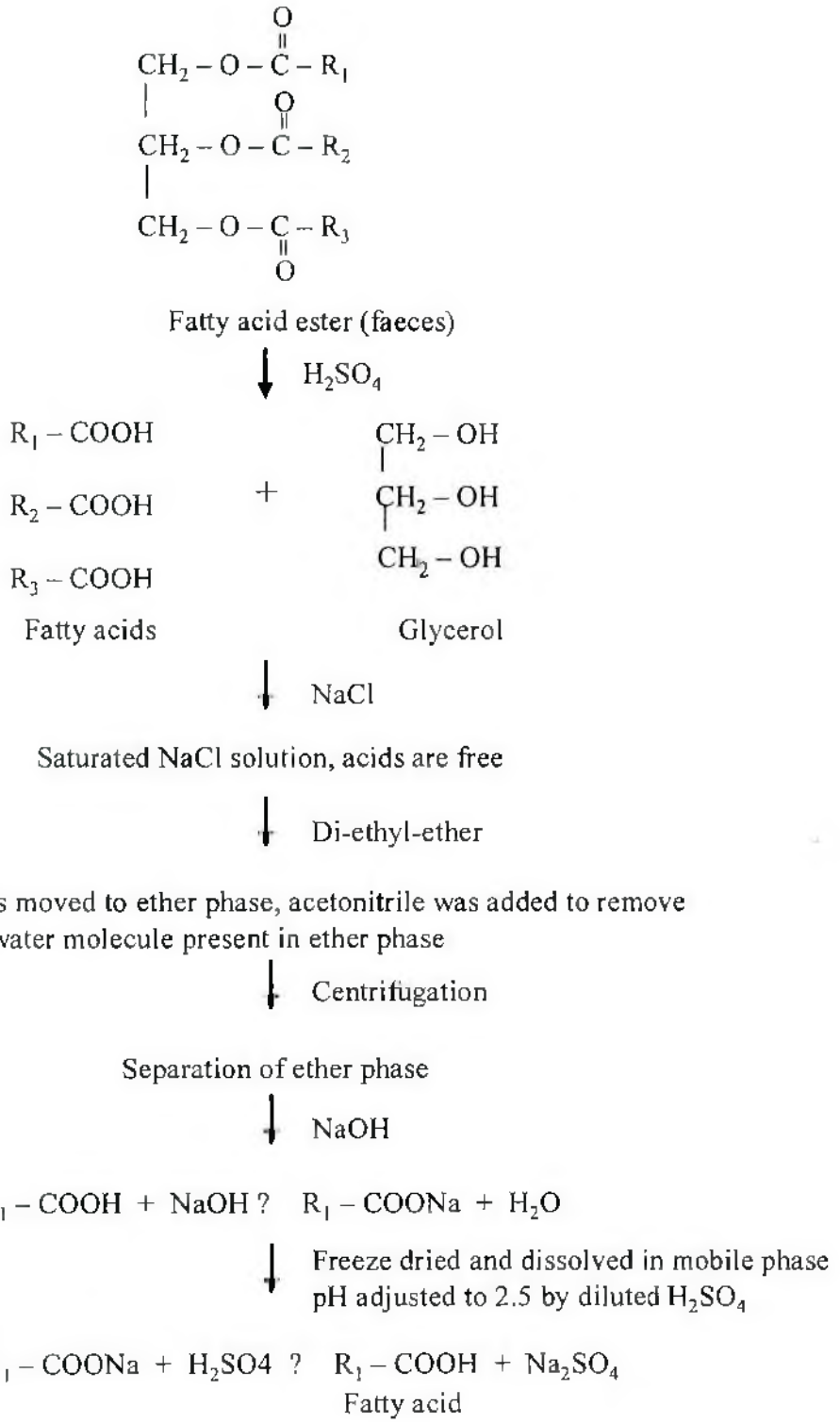


Figure 16. Flow diagram of faecal fatty acids extraction.

4.11.1 Instruments

- Centrifuge
- Vortex
- Electric balance

4.11.2 Reagents:

- Diethyl ether, CH_3-CH_2-O-CH_2-CH_3
- Sulphuric acid (18M), H_2SO_4
- Sodium chloride, NaCl
- Acetonitrile, CH_3CN
- Sodium hydroxide, NaOH

4.11.3 Procedure:

SCFAs were extracted from faecal samples using diethyl ether and the method was adapted from Guerrant *et al.*(47).

- One ml of watery stool was pipetted into a screw-capped falcon tube and the following reagents were added: 0.2 ml of 18 N H_2SO_4, 0.6 g of NaCl, 5 ml of diethyl ether (Merck KGaA, 64271 Darmstadt, Germany) and 25 µl of acetonitrile (HPLC Grade, Aldrich, Milwaukee, WI 53201, USA).
- The mixture was blended with a Vortex mixer for 8-10 minutes to mix the ether and aqueous phases.
- For semisolid and solid stools, 1g (wet weight) was suspended in the same reagents and blended.
- The tube was centrifuged at 1000 x g for 1 minute and the ether layer was transferred to another tube with a Pasteur pipette.
- 2-3 ml of diethyl ether was added to the residue again, mixed well and centrifuged as previous step. The ether layer was transferred to the previous tube with Pasteur pipette.

- To the ether, 0.2 ml of 0.1 N NaOH was added and the tube was gently shaken. With a Pasteur pipette, a small portion of the lower NaOH phase was removed and tested with pH paper. If necessary, 1 N NaOH was added, one drop at a time to obtain a pH of 9 or greater.
- The tube was blended with a Vortex mixer for 1 min and centrifuged and the upper ether phase was removed and discarded.
- 25 µl of acetonitrile was then added, and the tube was left uncapped for about 5 minutes to allow residual ether to evaporate.
- The NaOH phase was transferred in an eppendorf tube and freeze dried. The pellet was resuspended in mobile phase (10.8% acetonitrile in 0.007 N H_2SO_4), pH was adjusted to 2-3 by dilute H_2SO_4 (IM) and the volume was made 1 ml. The mixture of free acids was filtered through 0.2 µm membrane and 10 µl was introduced into the liquid chromatograph for analysis.

4.11.4 Standard Acids

Standard acetic, propionic, butyric and valeric acid (1g/ml for each acid) was purchased from Matreya, Inc. Pleasant gap P.A 16823 USA and were diluted in 0.007 N H_2SO_4 to prepare standard stock solution of five different concentration as follows 0.02 M, 0.01 M, 0.005 M, 0.0025 M, 0.00125 M. Stock solutions were preserved in 4° C. Five dilutions of each acid were run in HPLC to prepare the standard curve (Figure 17) from which the concentration of study children faecal SCFAs was calculated. Figure 18 is presented a typical chromatogram containing peaks from four standard acids.

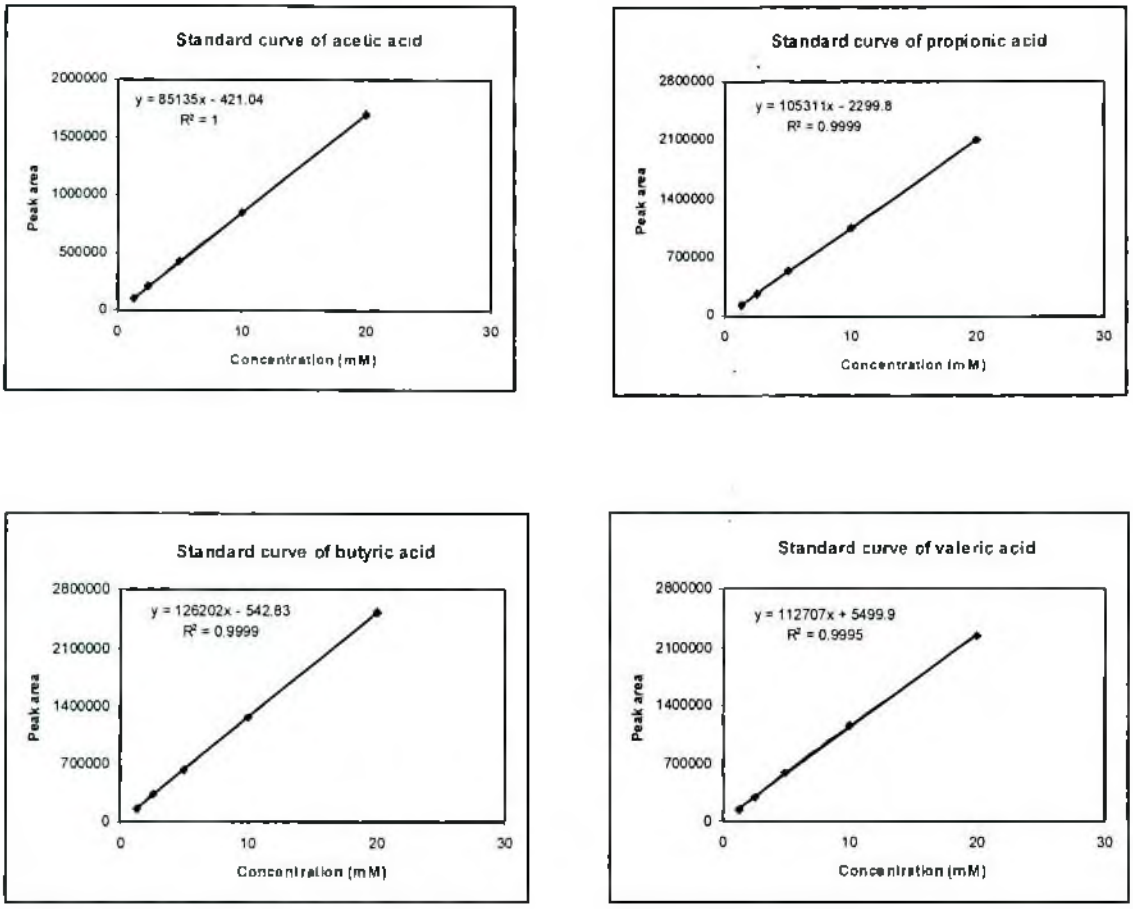
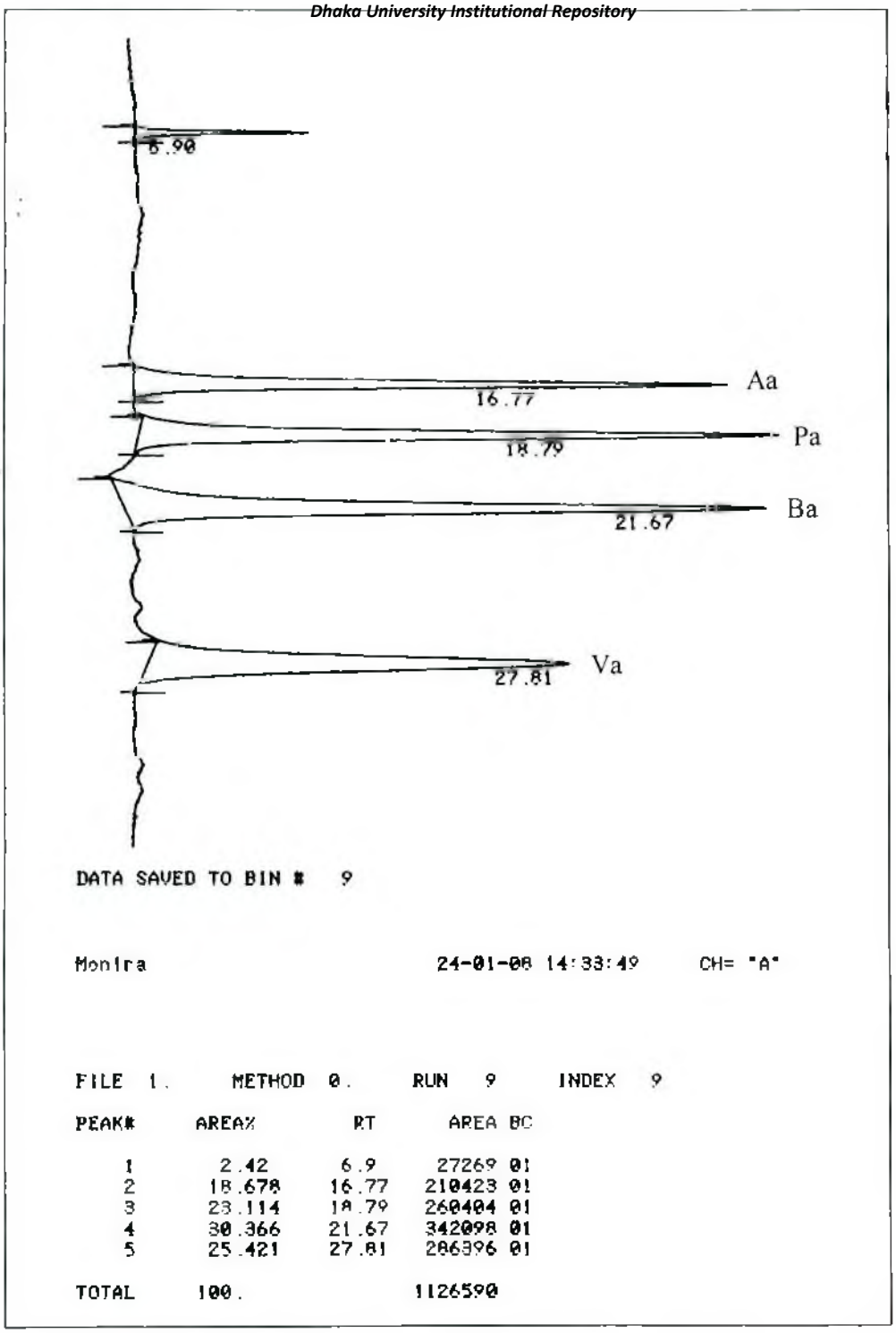


Figure 17. Standard curve of acetic acid, propionic acid, butyric acid and valeric acid.



DATA SAVED TO BIN # 9

Monira 24-01-08 14:33:49 CH= "A"

FILE 1. METHOD 0. RUN 9 INDEX 9

PEAK#	AREA%	RT	AREA BC
1	2.42	6.9	27269 01
2	18.678	16.77	210423 01
3	23.114	18.79	260404 01
4	30.366	21.67	342098 01
5	25.421	27.81	286396 01
TOTAL	100.		1126590

Figure 18. Chromatogram of four standard acids (2.5 mM) showing the retention time and peak area.
Aa, Acetic acid; Pa, Propionic acid; Ba, Butyric acid; Va, Valeric acid.

4.11.5 Liquid chromatograph and it's accessories

The High Performance Liquid Chromatography (HPLC) equipment used was composed of a pump (LC-10AT, VP Liquid Chromatograph, Shimadju Corp., Japan), a detector (SPD-10AV, UV-Vis Detector, Shimadju Corp.) and an integrator (Chromatopac, C-R6A, Shimadju Corp.). The chromatograph was equipped with an Aminex HPX-87H cation-exchange column (300 by 7.8 mm) for organic acids (125-0140; Bio-Rad Laboratories, Richmond, Calif.), a Micro-Guard- column (125-0129; Bio-Rad), and a holder (125-0131; Bio-Rad). A 50 µl bypass sampling loop was used, and the Detector was adjusted to a wavelength of 210 nm and to a sensitivity of 0.01 absorbance units (full scale). Ambient temperature was used and the flow rate was 0.5 ml /minute using column eluent composed of 10.8% acetonitrile in 0.007 N H_2SO_4 (0.19 ml of H_2SO_4 per litre) with a chart speed of 5, attenuation 5 and stop time of 40 minutes. The eluent was degassed using a sonicator before using.

4.12 Determination of faecal microbiota structure by fluorescent in situ hybridization (FISH) combined with Flow cytometry

In situ is a Latin phrase meaning "*in the place*". In situ means to examine the phenomenon exactly in place where it occurs (i.e. without any modification or moving it to some special medium). *In situ* hybridization was introduced in 1969 (35) is a highly sensitive technique that allows detection and localization of specific DNA or RNA molecules in morphologically preserved cells, histological tissue sections, or chromosomal preparations. It is a powerful technique and unique in the way that it allows studying the macroscopic distribution and cellular localization of DNA and RNA sequences in a heterogeneous cell population. Figure 19 shows the major steps involved in situ hybridization : oligonucleotide probe preparation and labeling by isotope or fluorescent dyes, cell fixation by ethanol or paraformaldehyde,

permeabilization of cell by lysozyme, hybridization, and signal detection by autoradiography or fluorescence microscope or flow cytometry (141). The basic technique utilizes the fact that DNA and RNA will undergo hydrogen bonding to complimentary sequences of DNA.

In situ identification and enumeration of microorganisms harbours a technique in which a certain rRNA sequence is specifically detected within morphologically intact cells, referred to as whole-cell hybridization (12).

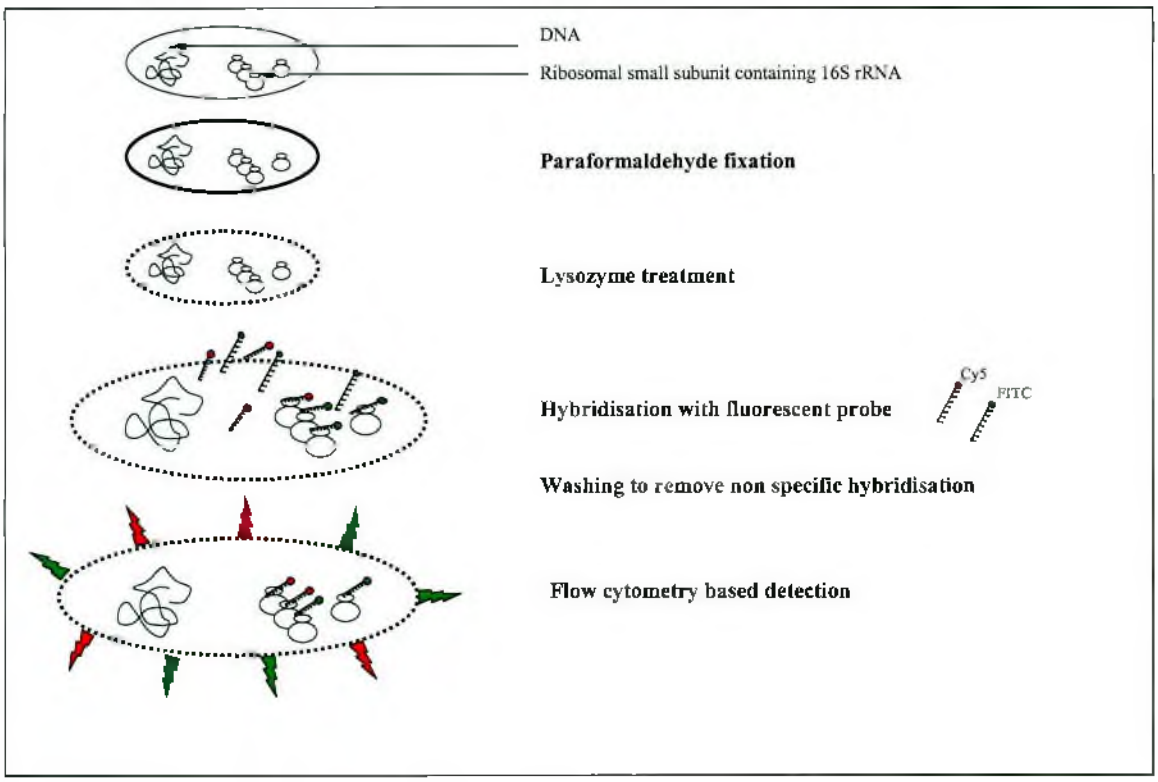


Figure 19. Principle of Fluorescent in situ hybridization.

Use of this technique for counting and identifying of microorganisms was first proposed by Olsen *et al.* (144). At the beginning age of in situ hybridization, the microscopic identification of single microbial cells with rRNA-targeted probes was performed with radioactively labeled oligonucleotides (80). Fluorescent probes yield superb spatial resolution and instantaneously detected by epifluorescence microscopy. Fluorescently monolabeled, rRNA-targeted oligonucleotide probes demonstrated the detection of individual cells (49). This made whole-cell hybridization with rRNA-targeted probes a suitable tool for determinative, phylogenetic, and environmental studies in microbiology (11). Like immunofluorescence, whole-cell hybridization with fluorescently labeled rRNA-targeted

oligonucleotides can be combined with flow cytometry for a high-resolution automated analysis of mixed microbial populations (212).

4.12.1 Permeabilization of bacterial cells

The following reagents were needed : Lysozyme (Sigma, St. Louis, MO, USA), Tris-EDTA, PBS, Hybridization buffer.

The objective of the permeabilization by lysozyme treatment is to facilitate the entrance of the probe inside the bacterial cell. 50 µl of faecal suspension fixed by paraformaldehyde was permeabilized by Tris-EDTA buffer containing 1 mg ml^{-1} lysozyme and incubated for 10 min at room temperature. Cells were then washed in PBS to remove lysozyme and equilibrated in 400 µl hybridization solution for enumeration of bacterial number. For identification of bacterial proportion by group specific probe, another 50 µl fixed faecal suspension was taken from the mother stock vial and permeabilized in the same way and finally dissolved in 700 µl of hybridization buffer.

4.12.2 Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells when they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. These signals are separated by optical filters and collected by photomultipliers, amplified, digitized, treated and stored in a computer. This technology of individual analysis (cell by cell) is multiparametric and can be made at a speed of more than several thousand of events per second. The computer will calculate statistical data associated to the measured parameters (Figure 20).

The flow cytometry is a technology which was used at the beginning to characterize the blood cells. This technology covers a wide range of research field and disciplines (plant

biology, zoology, microbiology, marine ecology, embryology, immunology, genetic, molecular biology and clinical diagnostic). It has been recently applied and adapted to the study of the microbial ecosystems (12). This technology is a rapid tool of analysis and is well adapted to the study of the digestive microbiota from healthy adults (160, 161). The flow cytometry has the advantage to analyse in less than 30 seconds more than 100,000 cells (representing 1,000 to 2,000 microscopic fields). Another advantage is the fastness (less than 10 minutes per sample).

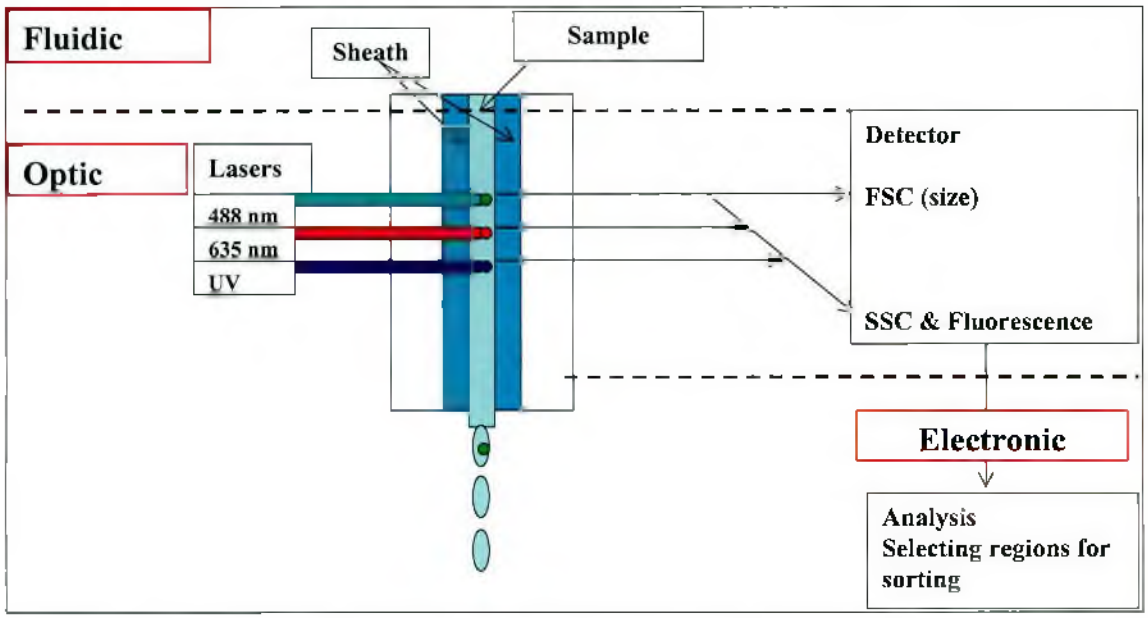


Figure 20. Schematic representation of flow cytometry equipment.

Data acquisition was performed using a FACS Calibur flow cytometer (Becton Dickinson, USA) equipped with an air cooled argon ion laser providing 15mW at 488 nm combined with a 635 nm red-diode laser. All the parameters were collected as logarithmic signals. The 488 nm laser was used to measure the forward angle scatter (FSC, in the 488 nm band-pass filter), the side-angle scatter (SSC, in the 488nm band-pass filter), and the green fluorescence intensity (FL1, in the 530 nm band-pass filter) conferred by the FITC-labeled probes. The red diode laser was used to detect the red fluorescence conferred by the Cy5 labeled probes (FL4, in a 660 nm band-pass filter). The acquisition threshold was set in the side scatter channel. The rate of events in the flow was generally 7000 events s^{-1}. With faecal suspensions, a total of 100 000 events were stored in the list modes files.

4.12.3 Enumeration of bacterial cells

For enumeration, 200 µl of cell suspension was taken in each of two flow cytometry tube. In one tube, 10 µl fluorescent particle suspension (High Intensity Alignment Grade Particles, 6.0 µm, Polysciences, Eppelheim, Germany) and 2.5 µl propidium iodide was added and in another tube only 2.5 µl propidium iodide was added. 100 µl of PBS was added in each tube, waited for 10 min and data acquisition was done by Flow cytometry (115).

4.12.3.1 Calculation:

Calculations were conducted using Cell Quest software (Becton Dickinson). For each stool sample, the total number of bacteria was calculated from a ratio of the event number derived from bacterial cells compared with a known number of internal standard fluorescent particles added to each duplicate tube. Consequently, the volume was analysed using the flow cytometer based on the number of particles and the bacterial concentration was calculated in the stool samples.

At the beginning, the calculation was standardized using a suspension made of *E. coli* strain ATCC 25922. An 18-hour culture of *E. coli* 25922 was fixed by paraformaldehyde similar as faecal sample. Diluted suspension was prepared containing 4.0×10^8 cells/ml by measuring the absorbance in spectrophotometer at 600 nm. This suspension was permeabilized by lysozyme, two alliquoted tubes were prepared and data acquisition was done by Flow cytometry as described before. The data presented by dot plot and histogram with statistics in flow cytometer are shown in Figure 21 and 22.

R1 region is defined as the area of dust and fluorescent particle.

R2 region is defined as the bacterial area.

The concentration of fluorescent particle in stock vial was 2×10^6 particle/ml.

Data in Figure 21 is without particle and in 22 was with particle.

So, the total number of dust and particle is = 194 (R1, Figure 22)

The number of dust is = 2 (R1, Figure 21)

i.e., the number of particle is = 192

The calculated particle volume is = 2.97 µl.

From R2 region in Figure 22, the bacterial number is = 93703

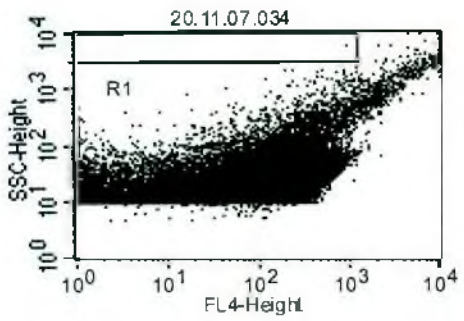
The bacterial concentration is = 31478.35

In flow cytometer tube, the bacterial number is = 9758288.98 (310 µl)

In 50 µl fixed bacterial suspension, number is = 19516577.9

So, the concentration of bacteria is = 390331559.37

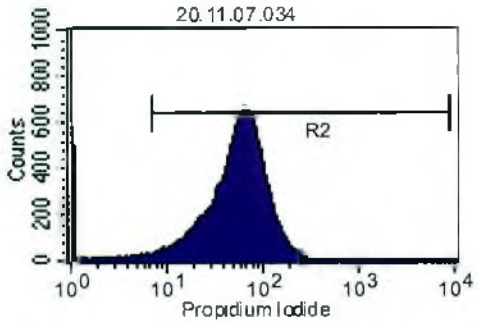
$= 3.9 \times 10^8$ / ml



Region

File: 20.11.07.034 Log
Sample ID: Pati
Tube: Untitled Pan
Acquisition Date: 01-Jan-70 Gat
Gated Events: 100000 Tot
X Parameter: FL4-Height (Log) Y P

Region	Events	% Gated	% Total	X Me
R1	2	0.00	0.00	774
R2	93535	93.53	93.53	222

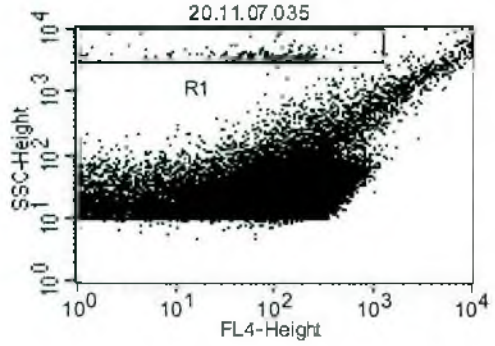


Histo

File: 20.11.07.034
Sample ID:
Tube: Untitled
Acquisition Date: 01-Jan-70
Gated Events: 100000
X Parameter: Propidium Iodide (Log)

Marker	Left, Right	Events	% Gated	%
All	1, 9910	100000	100.00	1

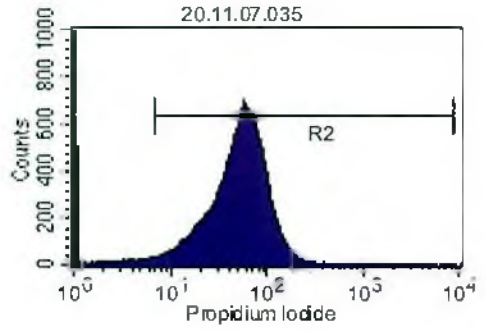
Figure 21. Enumeration of bacterial cell by flow cytometry in faecal sample without fluorescent particle.



Region Sta

File: 20.11.07.035 Log Dal
Sample ID: Patient
Tube: Untitled Panel: L
Acquisition Date: 01-Jan-70 Gate: N
Gated Events: 100000 Total E
X Parameter: FL4-Height (Log) Y Paran

Region	Events	% Gated	% Total	X Mean
R1	194	0.19	0.19	106.70
R2	93703	93.70	93.70	200.77



Histogra

File: 20.11.07.035 L
Sample ID: P
Tube: Untitled P
Acquisition Date: 01-Jan-70 G
Gated Events: 100000 T
X Parameter: Propidium Iodide (Log)

Marker	Left, Right	Events	% Gated	% To
All	1, 9910	100000	100.00	100.(

Figure 22. Enumeration of bacterial cell by flow cytometry in faecal sample with fluorescent particle.

4.12.4 Detection of bacterial groups by probes targeted against 16S rRNA

The method was validated in Department of Biology, CNAM, Paris. Reference bacterial strains specific to each probe e.g., *Escherichia coli* (ATCC 11775), *Clostridium coccoides* (ATCC 29236), *Bacteroides vulgatus* (ATCC 8482), *Faecalibacterium prausnitzii* (ATCC 27766), *Bifidobacterium longum* (ATCC 15707), *Collinsella aerofaciens* (ATCC 25986), *Lactobacillus acidophilus* (ATCC 4356) and *Enterococcus faecalis* (CIP 76117) were tested in that laboratory to check the hybridization step and probes specificity. The same experiment protocol was followed for faecal bacterial group identification in Laboratory Sciences Division, ICDDR, B, Dhaka.

50-µl aliquot of hybridized suspension was used for FISH with control and group-specific probes targeting the small subunit of rRNA. The Eub 338 probe and NON-Eub 338 probe were used as the positive control and negative control respectively. Both control probes were covalently linked at their 5' end either to fluorescein isothiocyanate (FITC) or to the indodicarbocyanine (Cy5). The group-specific probes were only labeled at their 5' end with Cy5. Hybridization was performed in a 96-well microtitre plate overnight at 35° C in the Hyb solution containing 4 ng μl^{-1} of the appropriate labeled probe(s). The probe sequences and targeted groups are presented in Table 12. Following hybridization, 150 µl of hybridization solution was added in each well and the cells were pelleted at 4000 X g for 15 min. Non-specific binding of the probe was removed by incubating the bacterial cell suspension at 37°C for 20 min in a washing solution (64 mm NaCl, 20 mm Tris-HCl, pH 8.0, .01% SDS). Cells were finally pelleted and resuspended in 200 µl PBS (pH 7.2). Alliquotes of 150 µl were added to 200 µl of PBS for data acquisition. The arrangement of hybridization tubes during data acquisition are presented in Table 13.

Table 12. DNA probes used for fluorescent in situ hybridization.

Probe	Target	Sequence from 5' to 3' end	OPD code	Reference
Eub 338	Bacteria	GCT GCC PCC CGT AGG AGT	S-D Bact-0338-a-A-18	(12)
NON Eub 338		ACA TCC TAC GGG AGG C	S-D Bact-0338-a-S-18	(212)
Enter 1432	Enterobacteriaceae family	GTT TTG CAA CCC ACT	S-G-Enter-1432-a-A-15	(185)
Erec 482	*Clostridium coccoides* group	GCT TCT TAG TCA GGT ACC G	S-*-Erec-0482-a-A-19	(78)
Bact 1080	*Bacteroides* group	GCA CTT AAG CCG ACA CCT	S-*-Bacto-1080-a-A-18	(57)
Fprau 645	*Faecalibacterium prausnitzii* subgroup	CCT CTG CAC TAC TCA AGA AAA AC	S-*-Fprau-0645-a-A-23	(198)
Bif 164	*Bifidobacterium* genus	CAT CCG GCA TTA CCA CCC	S-G-Bif-0164-b-A-18	(108)
Ato 291	*Atopobium* cluster	GGT CGG TCT CTC AAC CC	S-*-Ato-0291-a-A-17	(94)
Lab 158	*Lactobacillus-Enterococcus* group	GGT ATT AGC A(C/T) C TGT TTC CA	S-*-Lacto-0158-a-A-20	(95)

OPD – Oligonucleotide Probe Database.

Table 13. Arrangement of hybridization tubes during data acquisition.

Tube	*Combination*
1	Eub-338 FITC
2	Non-Eub FITC
3	Eub-338 Cy5
4	Non-Eub Cy5
5	Eub FITC/Non-Eub Cy5
6	Eub FITC/Enter 1432
7	Eub FITC/Erec 482
8	Eub FITC/Bact 1080
9	Eub FITC/Fprau 645
10	Eub FITC/ Bif 164
11	Eub FITC/Ato 291
12	Eub FITC/Lab 158

4.12.4.1 Calculation

Subsequent analyses were conducted using Cell Quest software (Becton Dickinson). Bacterial cell enumeration was performed by combining, in one hybridization tube, one group specific Cy5 probe with the Eub 338-FITC probe (tube 6 -12, Table 13). An FL1 histogram (Green fluorescence) was used to evaluate the total number of bacteria hybridizing with the Eub338-FITC probe. A gate was designed in this histogram representing the total number of bacterial cells in the sample and was used to build an FL4 histogram (Red fluorescence) to directly estimate the proportion of cells targeted by the group Cy5 probe in the sample. The proportion of cells was corrected by eliminating background fluorescence, which was measured using the negative control NON-Eub 338- Cy5 probe (tube 5, Table 13). Results were expressed as cells hybridizing with the group Cy5 probe as a proportion of the total bacteria hybridizing with the Eub 338-FITC bacteria domain probe (161). An example of

hybridization of universal bacterial probe and group specific bacterial probe to faecal bacteria of a patient at day 3 is presented in Figure 23 to 30.

4.13 Statistical analysis

The data were analyzed as means ± sem and the differences between the means were compared for significance using ANOVA (LSD, post hoc), and paired t-test. Standard curves for SCFAs were plotted by regression using least square method.

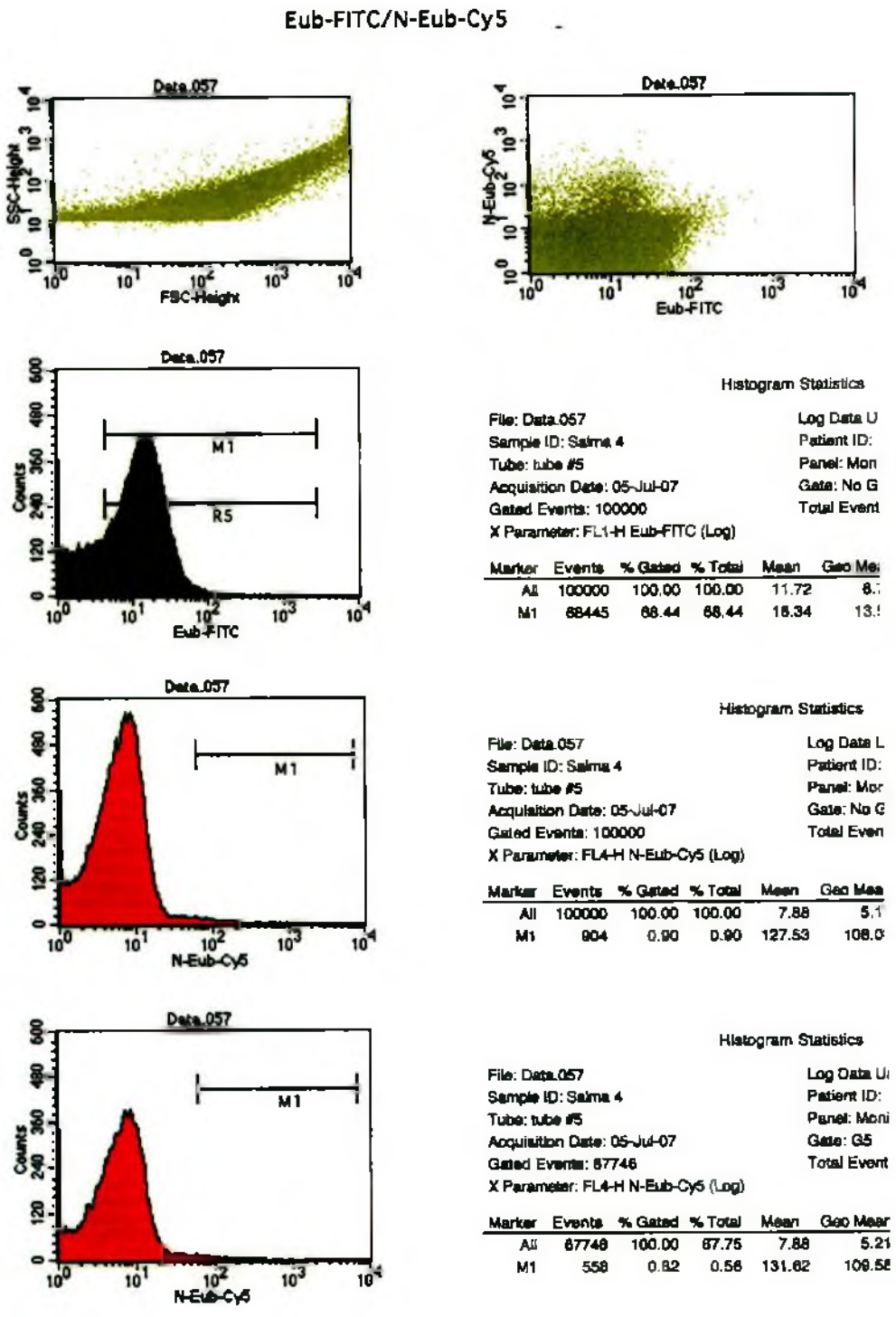


Figure 23. Hybridization of faecal bacterial cells by Eub-FITC/Non Eub-Cy5 probe in flow cytometry.

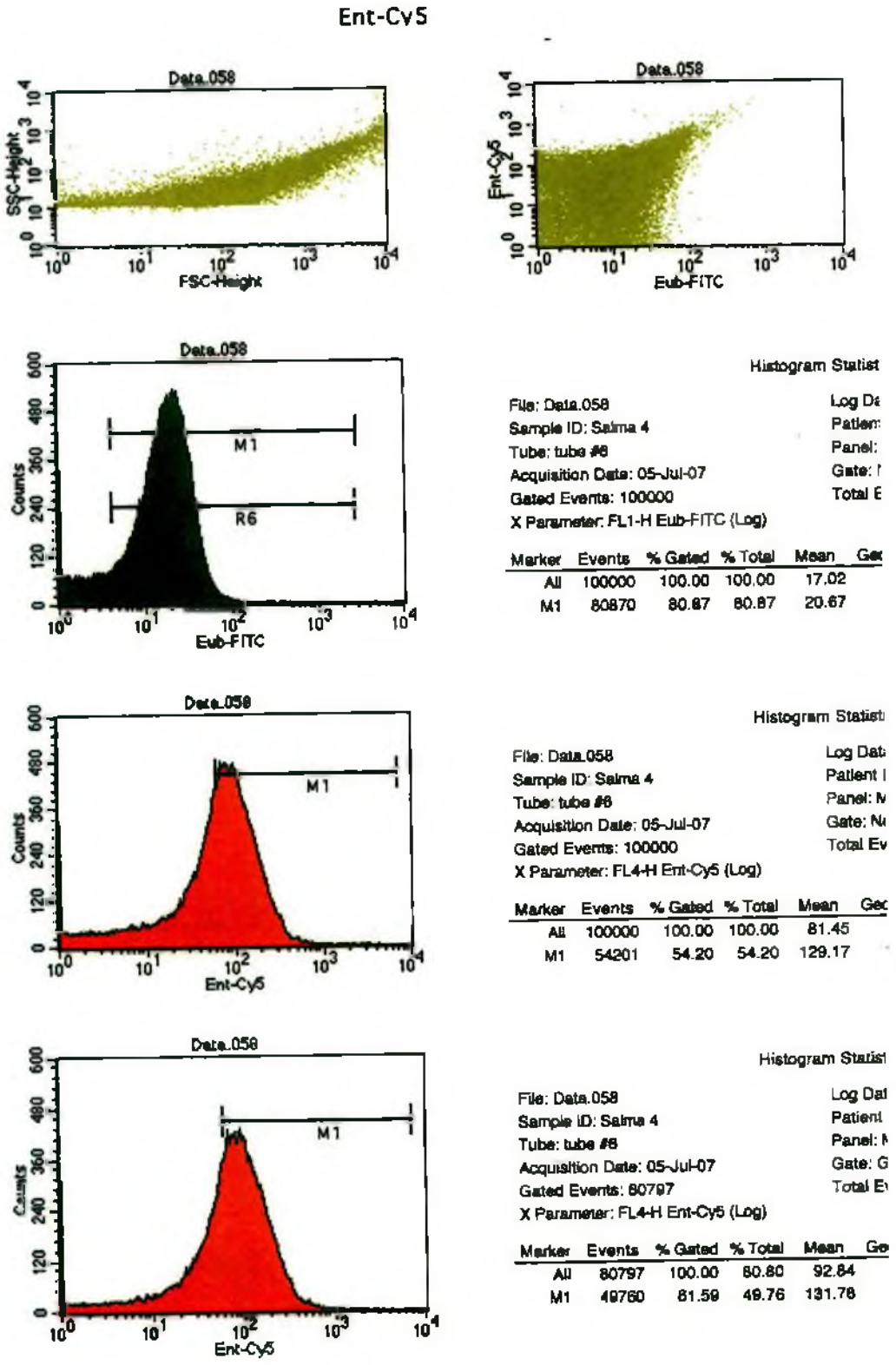


Figure 24. Hybridization of faecal bacterial cells by Eub-FITC/Ent-Cy5 probe in flow cytometry.

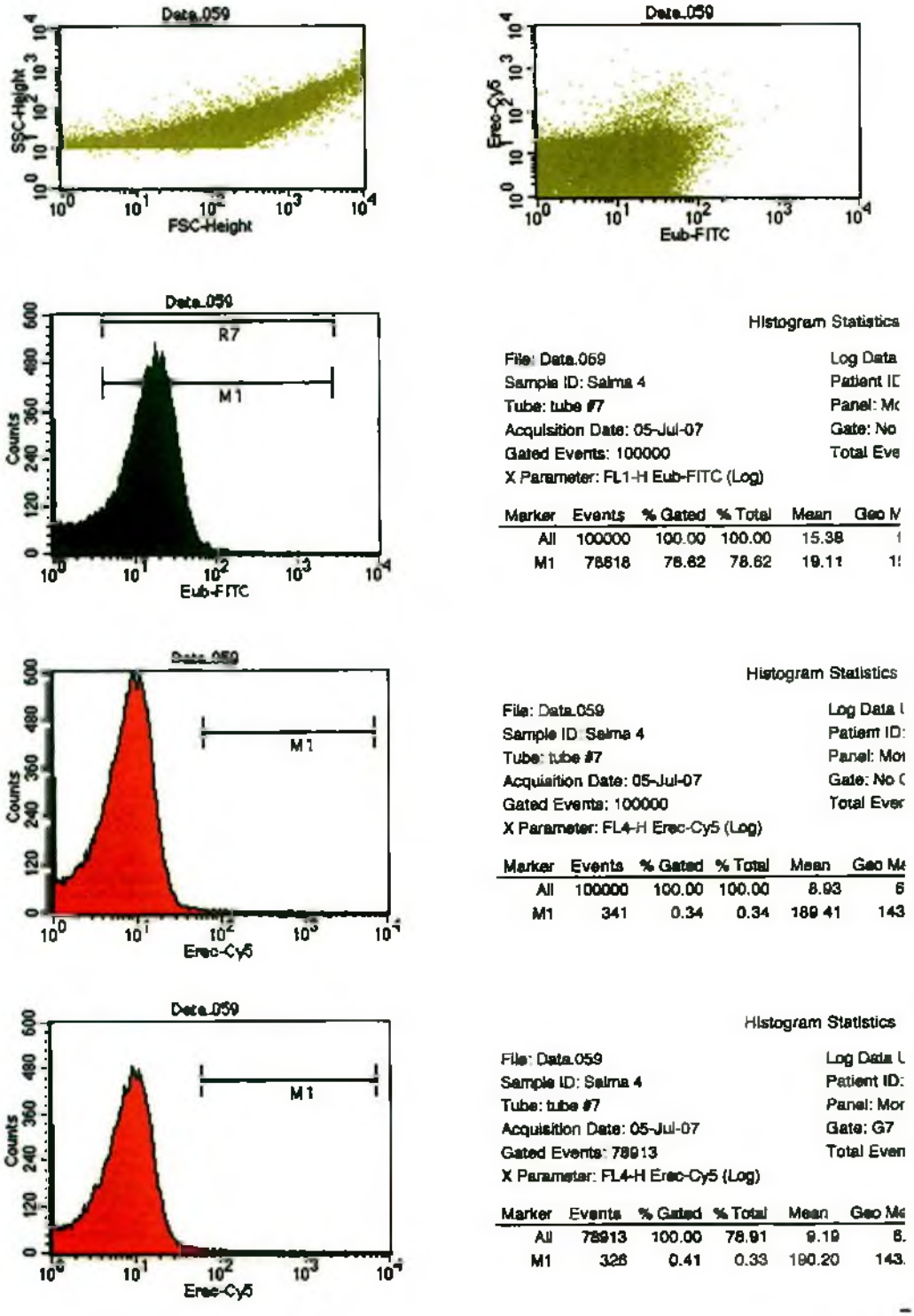


Figure 25. Hybridization of faecal bacterial cells by Eub-FITC/Erec-Cy5 probe in flow cytometry.

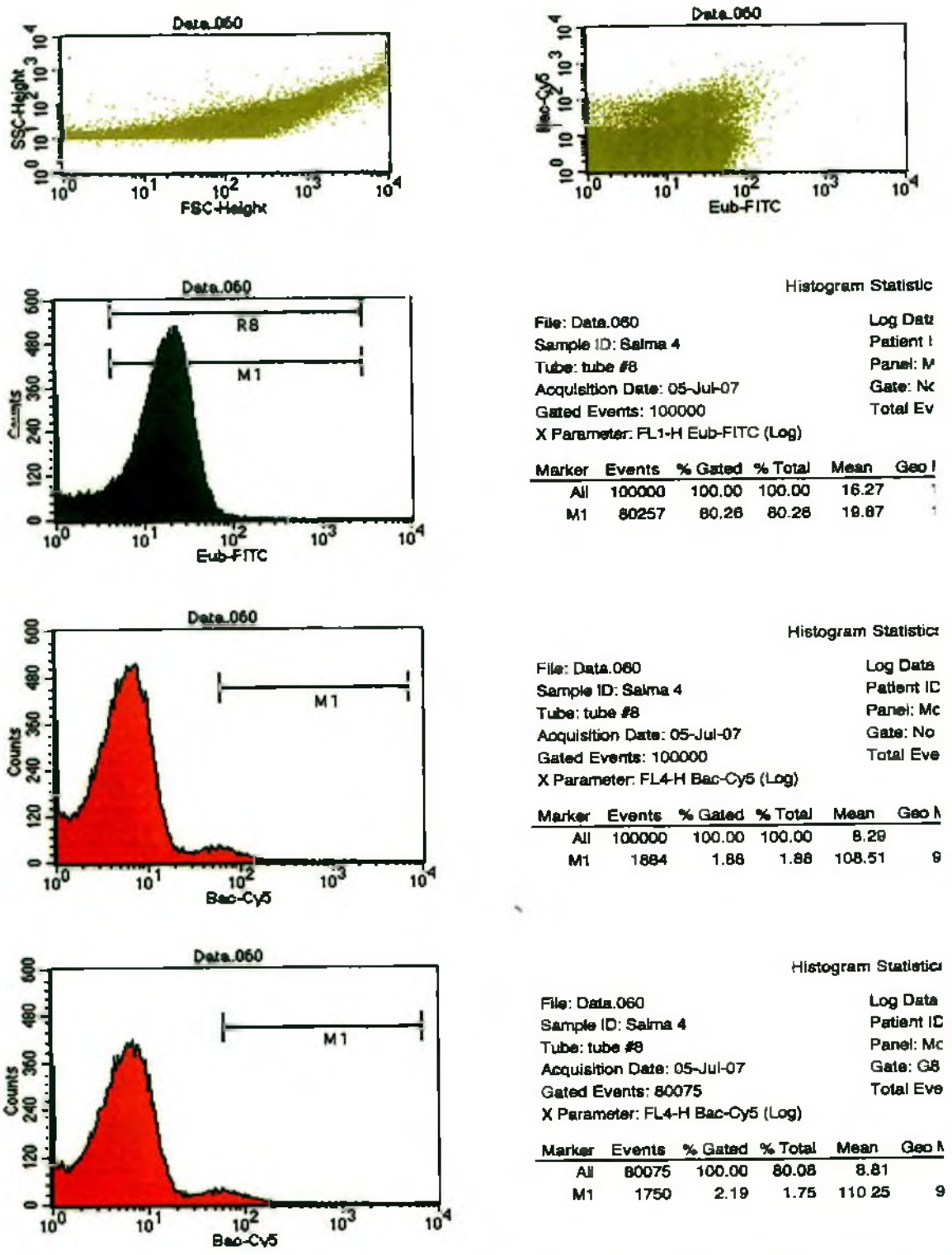


Figure 26. Hybridization of faecal bacterial cells by Eub-FITC/Bac-Cy5 probe in flow cytometry.

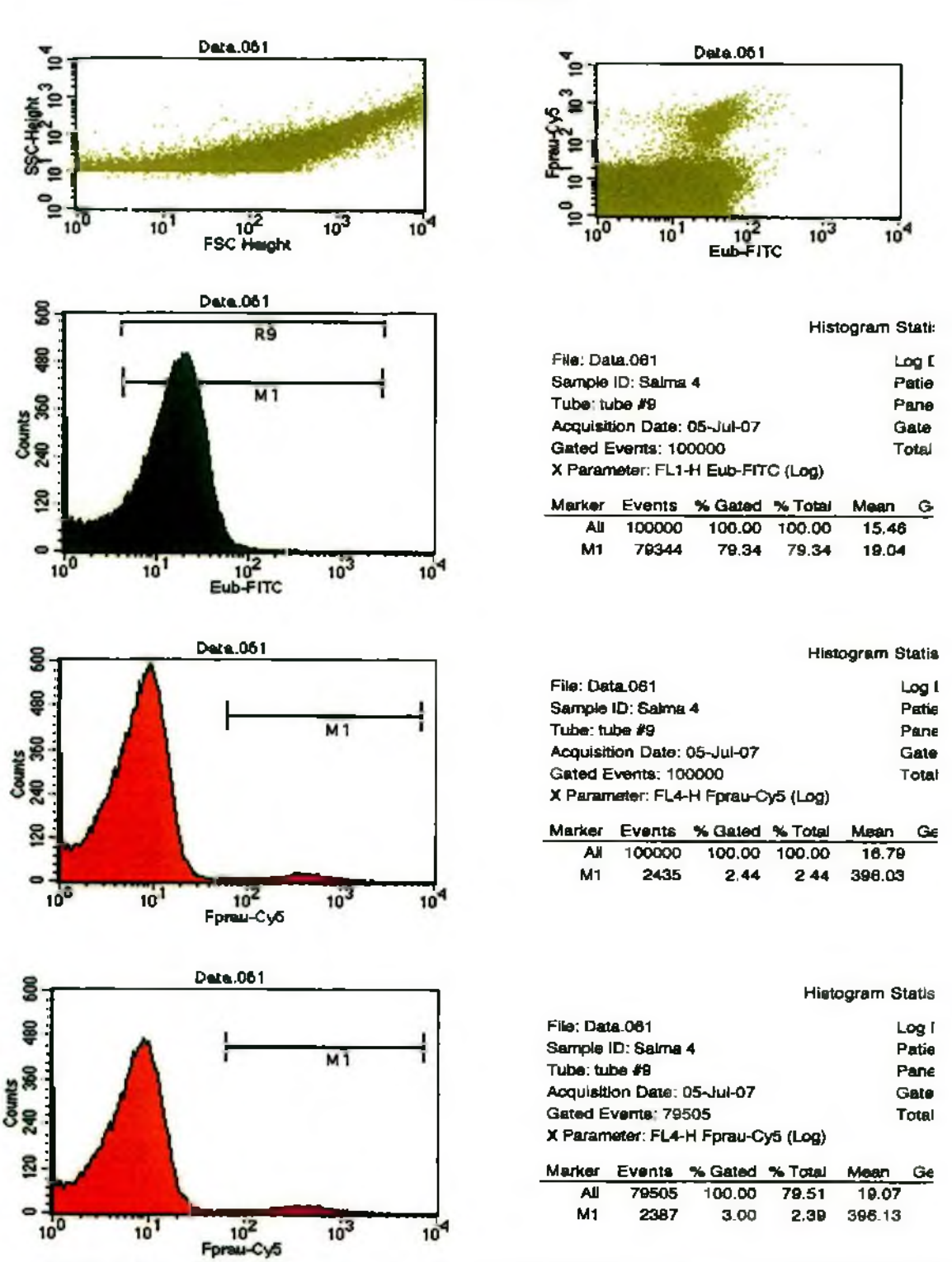


Figure 27 Hybridization of faecal bacterial cells by Eub-FITC/Fprau-Cy5 probe in flow cytometry.

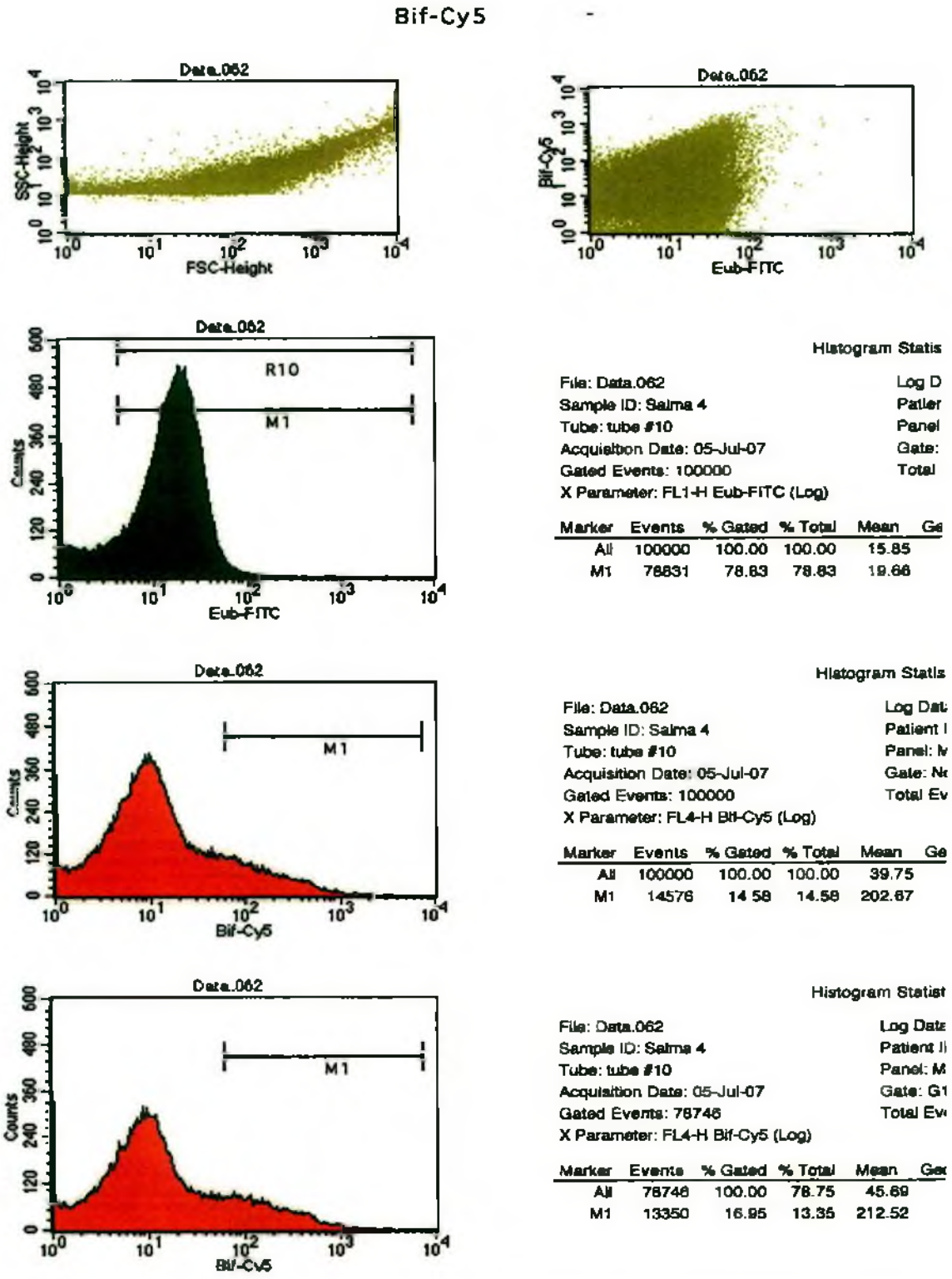


Figure 28 Hybridization of faecal bacterial cells by Eub-FITC/Bif-Cy5 probe in flow cytometry.

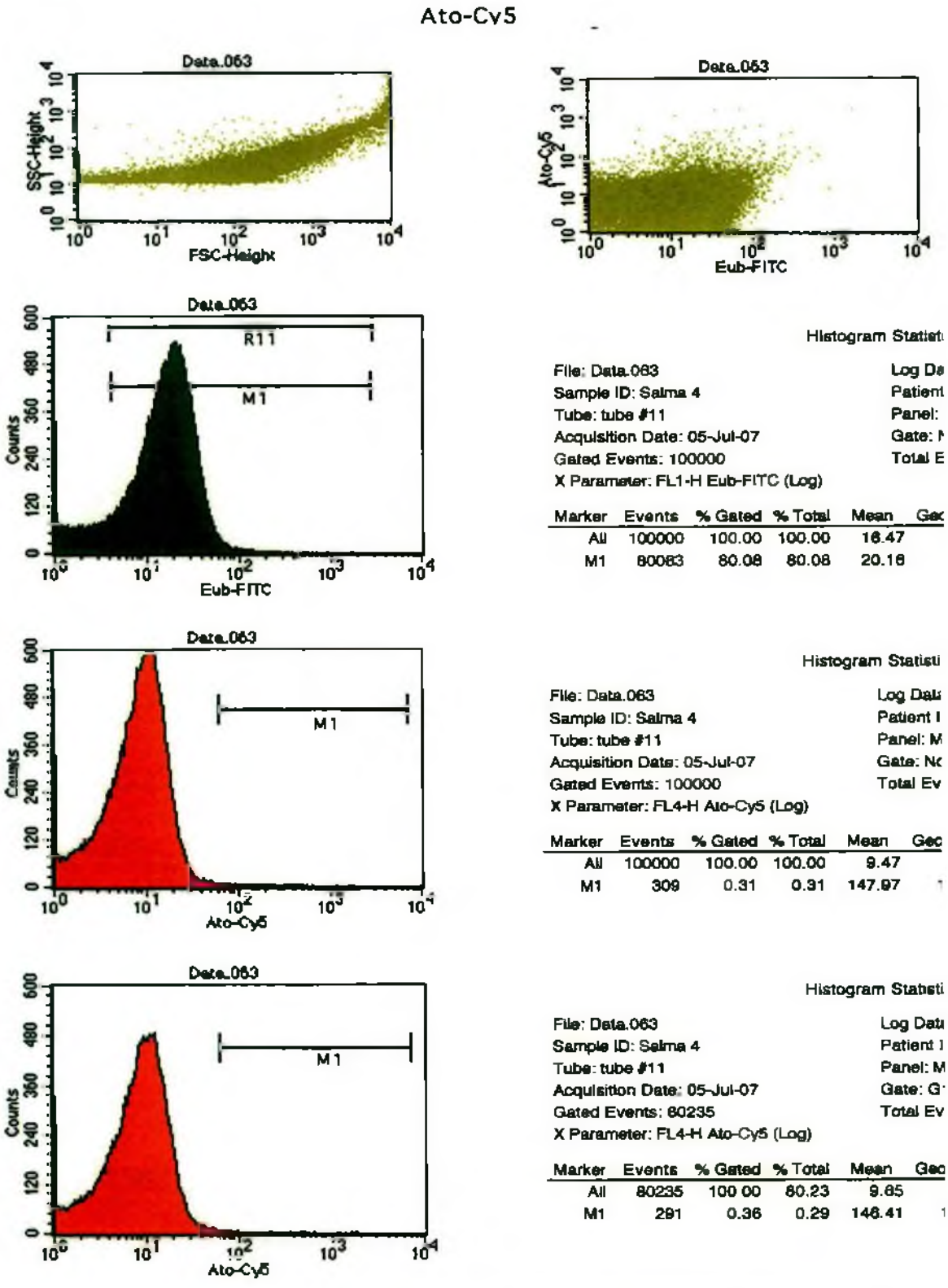


Figure 29 Hybridization of faecal bacterial cells by Eub-FITC/Ato-Cy5 probe in flow cytometry.

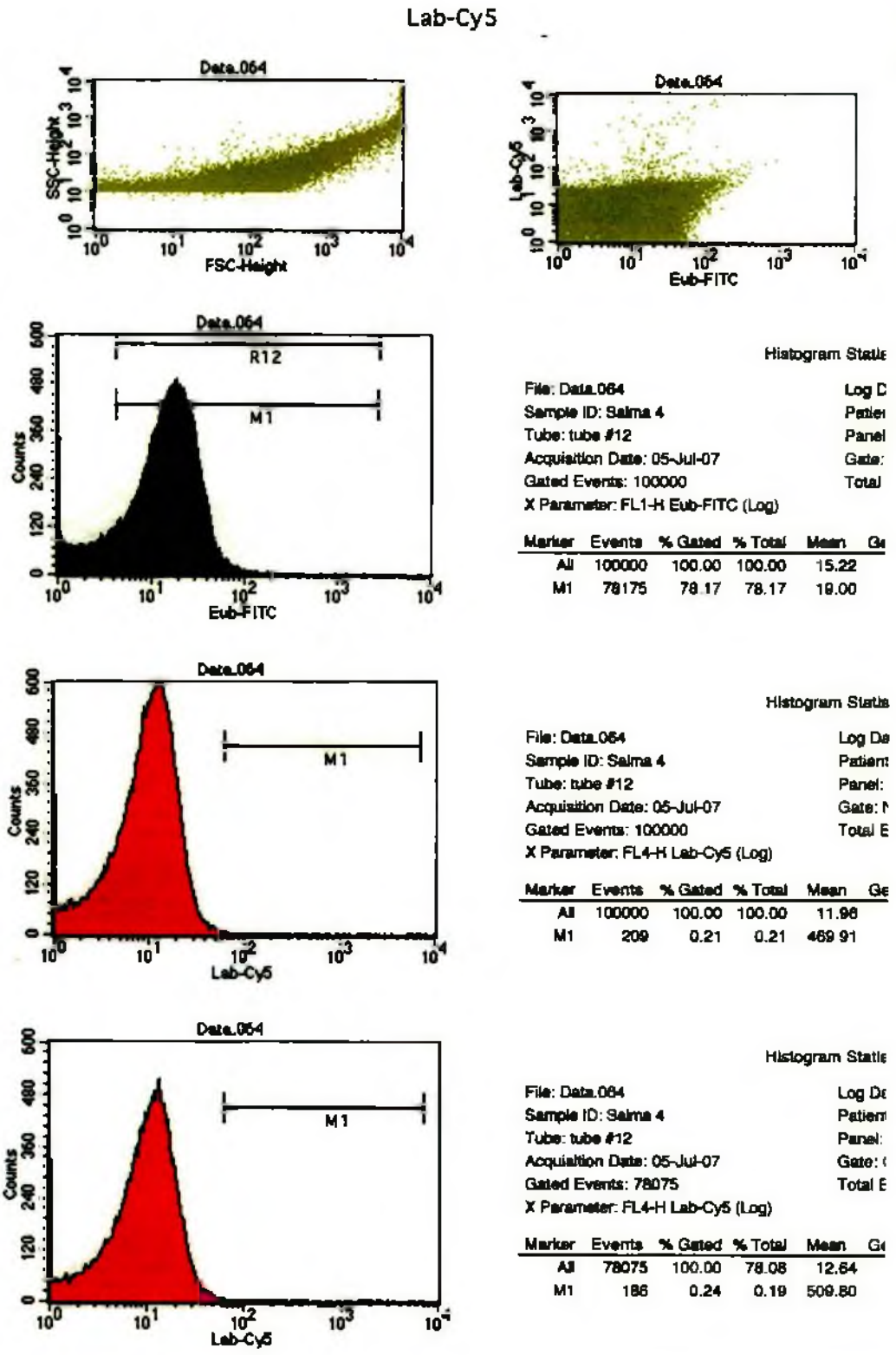


Figure 30. Hybridization of faecal bacterial cells by Eub-FITC/Lab-Cy5 probe in flow cytometry.

Chapter V

Results

5.0 Results

5.1 Baseline characteristics of the enrolled children

As a subset of the large clinical study, 30 severely malnourished children with cholera were randomly enrolled in the present study. For comparison, age matched 11 severely malnourished children without cholera and 6 healthy children were also enrolled. Their baseline characteristics were analysed and are presented in Table 14.

Table 14. Baseline characteristics of study children.

Children	Malnourished with cholera (N=30)			Malnourished without cholera (N=11)	Well-nourished without cholera (N=6)
ORS substrates	Glc	Glc+ARS	Rice	none	none
Age (month)	26.4 ±8.7	25.9 ± 9.0	29.8 ± 8.6	27.5 ± 4.1	27.7±3.9
Weight (kg)	7.2 ± 1.2	7.2 ± 1.3	7.4 ± 1.2	7.1 ± 0.4	12.3±1.3
Height (cm)	77.5 ±5.7	78.8 ± 6.6	80.2 ± 7.1	78.2 ± 3.5	84.2±2.9
Wt/Ht (%)	70.2 ±3.9	69.7 ± 4.1	66.1 ± 6.8	68.4 ± 1.4	106.5±5.8
Wt/Age (%)	57.6 ±6.8	59.2 ± 6.8	58.3 ± 5.8	55.1 ± 3.9	96.5±5.4
Male/Female	5/5	5/5	5/5	6/5	1/5

Values at inclusion are expressed as mean ± SD. N is number of children. Only the children with cholera received oral rehydration solution (ORS) with glucose (Glc), glucose and amylase resistant starch (Glc+ARS) or rice. Anthropometric measurements e.g., age, weight, height among the malnourished children of different groups were comparable. The weight and height parameters were different between well-nourished and malnourished children ($P<0.001$). The average duration of diarrhea and vomiting before admission was 12.2 ± 7.85 hour and 10.9 ± 7.17 hour respectively, (mean ± SD) and was not different among treatment groups. Only 48% of the subjects were reported to be breast feed before onset of illness.

5.2. Major clinical outcomes of the patients

Cholera children of rice ORS group had significantly reduced stool output and ORS intake than glucose ORS and glucose- ORS plus ARS group. Data presented in Table 15 and 16. The trend of stool output and ORS intake was also similar in the parent study. At 24 hours, compared to the glucose-ORS group, the reduction in stool output in rice-ORS group was 32%, at 48 hours 37%; and at 72 hours 36%. Similarly, the intake of ORS solution by the children in the rice-ORS group was also less when compared to glucose-ORS group: 27%, 37% and 38% at 24, 48 and 72 hours respectively. There was a trend in the reduction of stool output in the Glucose-ORS plus ARS group when compared to the glucose-ORS group; however, the difference was not statistically significant.

Table 15. Faecal weight in different treatment groups.

Period after enrollment	Faecal weight (g)			P value*		
	Glucose-ORS (N = 10)	Glc-ARS (N = 10)	Rice ORS (N = 10)	Glc. vs. ARS	Glc. vs. Rice	ARS vs. Rice
Day 0 – Day 1 or 0-24 hours	3817.7 ± 737.4	3162.3 ± 378.1	1941.6 ± 235.7	0.91	0.04	0.01
Day 1 – Day 2 25 to 48 hours	3151.9 ± 820.2	2171.5 ± 490.2	1104.9 ± 355.6	0.48	0.03	0.13
Day 2 – Day 3 49 to 72 hours	1074.6 ± 382.7	790.8 ± 219.7	326.5 ± 128.7	0.68	0.07	0.16

Values are presented as mean ± sem. *The mean was tested by ANOVA and post hoc test.

Table 16. ORS intake in different treatment groups.

Period after enrollment	ORS intake (ml)			P value*		
	Glucose-ORS (N = 10)	Glc-ARS (N = 10)	Rice ORS (N = 10)	Glc. vs. ARS	Glc. vs. Rice	ARS vs. Rice
Day 0 – Day 1	3311.8 ± 567.8	2909.0 ± 318.1	1883.3 ± 222.6	0.97	0.08	0.05
Day 1 – Day 2	3280.6 ± 894.7	2209.1 ± 545.4	993.9 ± 393.2	0.39	0.04	0.06
Day 2 – Day 3	1391.4 ± 465.8	921.2 ± 301.1	369.2 ± 189.9	0.43	0.04	0.18

Values are presented as mean ± sem. *The mean was tested by ANOVA and post hoc test.

Vomit and urinary output, and intake of water and infant formula did not differ statistically between three groups. The overall mean weight gain of the children at 72 hours was 114 (95% CI, 103; 124) g/kg and the gain were significantly greater in the rice-ORS group after 54 hours. The duration of diarrhea and the number of children receiving unscheduled intravenous therapy did not differ significantly between the groups. The overall mean duration of diarrhea was 66 hours. Finally, the end-point outcome i.e. the time to attain 80% of weight for length, counted from enrolment in the study, was not statistically different between the groups. None of the study children died during the study.

5.3 Biodiversity of faecal microbiota by Temporal Temperature Gradient Gel Electrophoresis (TTGE)

5.3.1 Diversity among malnourished children with cholera and without cholera

PCR-TTGE analysis of 16S rDNA was done to identify dominant bacterial species in the faecal samples of the study children. Such analysis of seven different faecal samples of each patient revealed various profiles comprising of 4 to 17 bands. A typical finding is presented in Figure 31a. In a first approximation and for the sake of communication, we assumed that each band corresponded to a different bacterial species. At day 0, before initiation of any treatment, the *V. cholerae* band was detected in all 30 patients, indicating its presence as the dominant bacteria at the time of acute cholera. The same band was found in only 5/30 (17%) children at day 1 and 1/30 (3%) children at day 2 after initiation of treatment, and then the band completely disappeared. In addition to the *V. cholerae* band, various other bands (mean ± sem, 7.7 ± 0.5) were observed at day 0. A rapid and significant increase of band numbers was observed at day 2 (day 1 vs. day 2, P = 0.002), followed by a steady increase observed up to day 28 (mean ± sem, 11.5 ± 0.6, range 9 to 17).

After resolution of cholera and partial recovery from malnutrition, the number of bands at day 28, was compared with the two other control groups of children. The mean ± sem number of bands in malnourished children with cholera was higher than those in malnourished children without cholera (11.5 ± 0.6 vs. 8.1 ± 1.1, P <0.027) but lower than that in healthy children (22.2 ± 0.4, P <0.01) (Figure 31 and Table 17). The range of bands in malnourished children without cholera and in healthy children was from 3 to 17 and from 20 to 24, respectively. The band number at day 0 (7.7 ± 0.5) is not different from band number of malnourished children without cholera (8.1 ± 1.1, P = 0.693) but significantly different from day 28 (P=0.001) and from healthy children (P< 0.01).

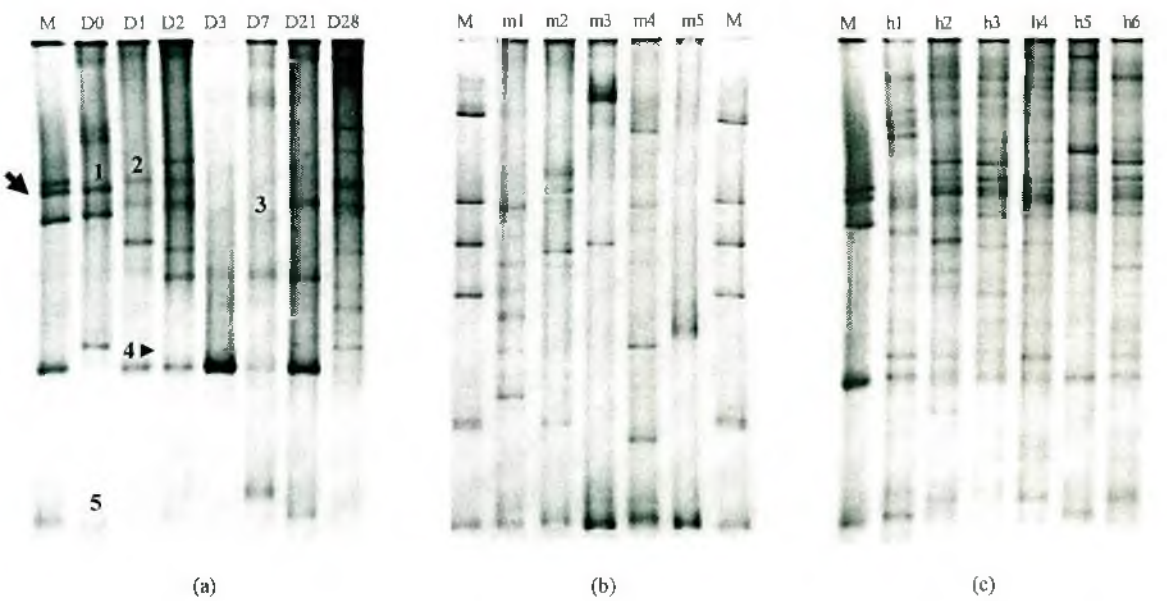


Figure 31. PCR- TTGE analysis of faecal bacterial populations from children with: a) malnutrition and cholera; b) malnutrition without cholera; and c) healthy.

In Figure 31a, the typical results of one patient were presented as function of time, from day 0 to day 28. Figure 31b and 31c presented the results of stool collected from 5 malnourished children (m1 to m5) and 6 healthy children (h1 to h6) on one day. The markers (M) used to calculate the Rf were identical in 31a and 31c, but different in 31b. (a) The arrow in the marker lane points to *Vibrio cholerae*. The bands labeled as 1, 2, 3, 4, 5 correlated sequentially the bacteria of the marker lane e.g., *V. cholerae*, *Prevotella sp.*, *E. facium*, *E. coli* and *Bifidobacterium longum*. However, for confirmation of bacteria belonging to each band sequencing is needed.

Table 17. Total number of bands obtained by PCR-TTGE from 16S rDNA in faecal microbiota of study children.

Group	Malnourished children with cholera (N=30)							Mal. Ch. (N=11)	Heal. Ch. (N=6)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	7.3 ± 0.7	6.3 ± 0.5	8.1 ± 0.6	7.3 ± 1.1	9.1 ± 0.9	9.1 ± 0.8	11.1 ± 0.4		
B (N=10)	7.4 ±0.9	7.4 ± 0.7	8.7 ± 0.8	10.6 ±0.9	9.8 ±0.6	9.7 ±1.0	11.7 ±1.0		
C (N=10)	8.4 ± 0.9	7.2 ± 0.9	9.2 ± 0.6	10.7 ± 1.2	10.6 ± 0.6	11.6 ± 1.1	11.6 ± 1.1		
Total (N=30)	7.7 ± 0.5	6.9 ± 0.4	8.7 ± 0.4	9.5 ± 0.7	9.8 ± 0.4	10.2 ± 0.6	11.5 ± 0.6	8.1 ±1.1	22.2 ±0.4
Significance (N = 30)	-	0.173	0.037	0.013	0.001	0.001	0.001	0.693	<0.001

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children. Significance was calculated by paired t-test between baseline value and others like D0 – D1, D0 – D2 and so on and between D0 - malnourished children and D0-healthy children at 95% CI.

5.3.2 Diversity among children with cholera treated with different ORS

During the first two days of treatment, no significant differences in the band numbers were observed between children receiving ORS containing different carbohydrates (Figure 32). On day 3, the number of bands was higher in rice-ORS and glucose-ORS plus ARS compared to those in glucose-ORS ($P < 0.05$). Afterwards, a trend for a higher number of bands was observed in patients with ORS containing complex carbohydrates (Rice or ARS) (Table 17).

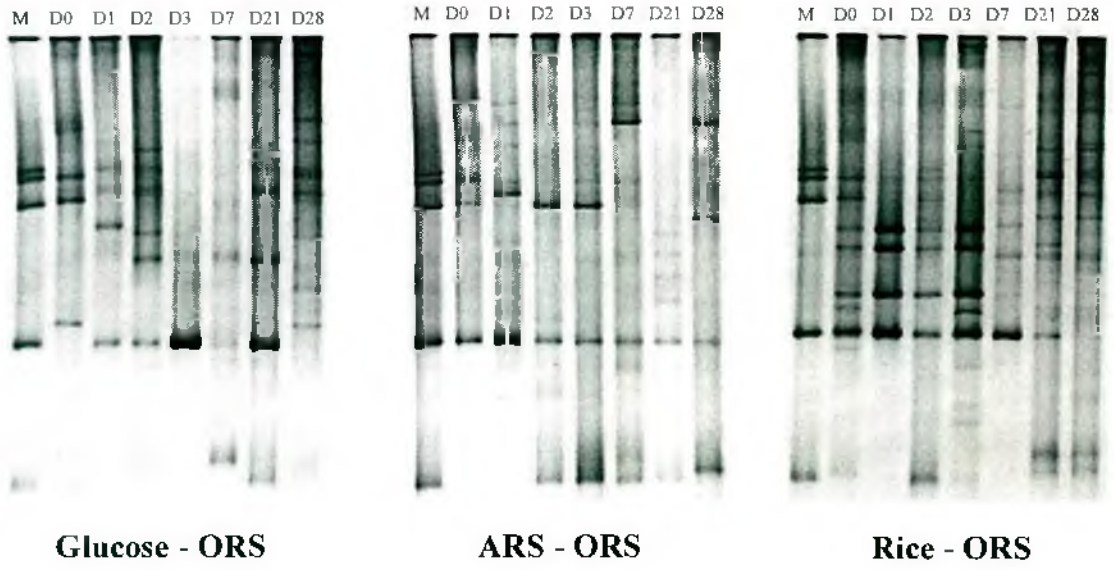


Figure 32. PCR — TTGE band profile of three representative patients treated with glucose-ORS, glucose-ORS plus ARS and rice ORS groups.

M, Marker for known bacterial species.

5.3.3 Evaluation of bacterial band profile similarity between different time periods of children with cholera

The TTGE profile of each patient was analyzed through Dice similarity coefficient in order to examine the re-establishment of microbiota in the gut ecosystem. Firstly, the distance of the samples were calculated from the first sample collected, e.g. between day 0 and day 1, between day 0 and day 2, and so forth. Secondly, the distance of bands between two consecutive samples were also calculated e.g. between day 0 and day 1, day 1 and day 2 and so forth. The mean values of these distances were analyzed to obtain a general picture of the faecal microbiota re-colonization in children with cholera. In the first set of analyses, TTGE profiles during the acute stage of cholera (day 0) was compared with recovering stages (day 1, day 2, day 3) and convalescent stages (day 7, day 21, day 28) and the average distances were calculated representing the colonization of faecal microbiota over these time periods. In the second set of analyses, the average distances between two consecutive samples were

compared to determine the speed of faecal microbiota evolution. Distances in both conditions were very high; indicating that the microbiotas were still evolving (Table 18).

Table 18. Distances in PCR-TTGE banding patterns of faecal microbiota from severely malnourished children with cholera.

Time Period (Days)						
Distance From Day 0	**D0-D1**	**D0-D2**	**D0-D3**	**D0-D7**	**D0-D21**	**D0-D28**
	(%)					
	80.9±20.2	86.8±14.5	90.0±15.4	86±14.4	84.6±14.1	87.5±13.3
Distance Between Days	**D0-D1**	**D1-D2**	**D2-D3**	**D3-D7**	**D7-D21**	**D21-D28**
	(%)					
	80.9±20.2	51.9±30.4	42.7±22.7	70.5±22.4	73.6±22.2	71.2±21.1

Values are means ± standard deviation. A distance between two days (D) of 100% represents no common bands, while 0% represents identity. The distance was measured as indicated in method section.

5.4 Sequence analysis of DNA from bands - cut of TTGE gel

In order to confirm that, the unknown DNA bands in patient sample those were parallel to marker bands (known bacteria) in TTGE gel are similar to the species or genus of the marker bacteria, DNA sequence of a few bands from several TTGE gel of different patients was determined and compared with the DNA sequence of markers in order to get bacterial identity. DNA sequence of total 11 bands (two bands parallel to each marker bands and one band parallel to lower band of *V. cholerae)* was determined and compared. A 449 base pair PCR product were sequenced and compared with public databases in Genbank by BLAST search. The results showed that, the sequences matched with bacterial species of the marker band by species or genus level with 92 – 100% similarity. They also matched with several clones of uncultured bacterium present in GenBank. One band parallel to *Prevotella sp.* did not match with genus or species level. Detailed data were presented in Table 19 .

Table 19. Comparison of sequences in GenBank obtained from DNA eluted from bands - cut of TTGE gel for identification of bacteria

Sl. No.	Band cut parallel to marker	Matched with first highest bit score			Matched with second highest bit score		
		Species/ Genus	Uncultured bacteria	Similarity (%)	Species/ Genus	Uncultured bacteria	Similarity (%)
1.	*Bifidobacterium longum* 1	Genus	+	100	-	+	99
2.	*Bifidobacterium longum* 2	Species	+	100	Species	+	99
3.	*Enterococcus facium* 1	Species	+	98	-	+	98
4.	*Enterococcus facium* 2	Genus	+	92	Species	+	92
5.	*V. cholerae, Upper band* 1	-	+	98	Species	+	98
6.	*V. cholerae,* Upper band 2	Species	-	99	Species	+	98
7.	*V. cholerae,* Lower band 1	-	+	96	Species	+	96
8.	*E. coli* 1	-	+	97	Species	-	97
9.	*E. coli* 2	Species	+	100	Genus	+	99
10	*Prevotella sp.* 1	-	+	87	-	+	86
11.	*Prevotella sp.* 2	-	+	100	Not found		

5.5 Analysis of faecal short chain fatty acids concentration

5.5.1 Total acid concentration

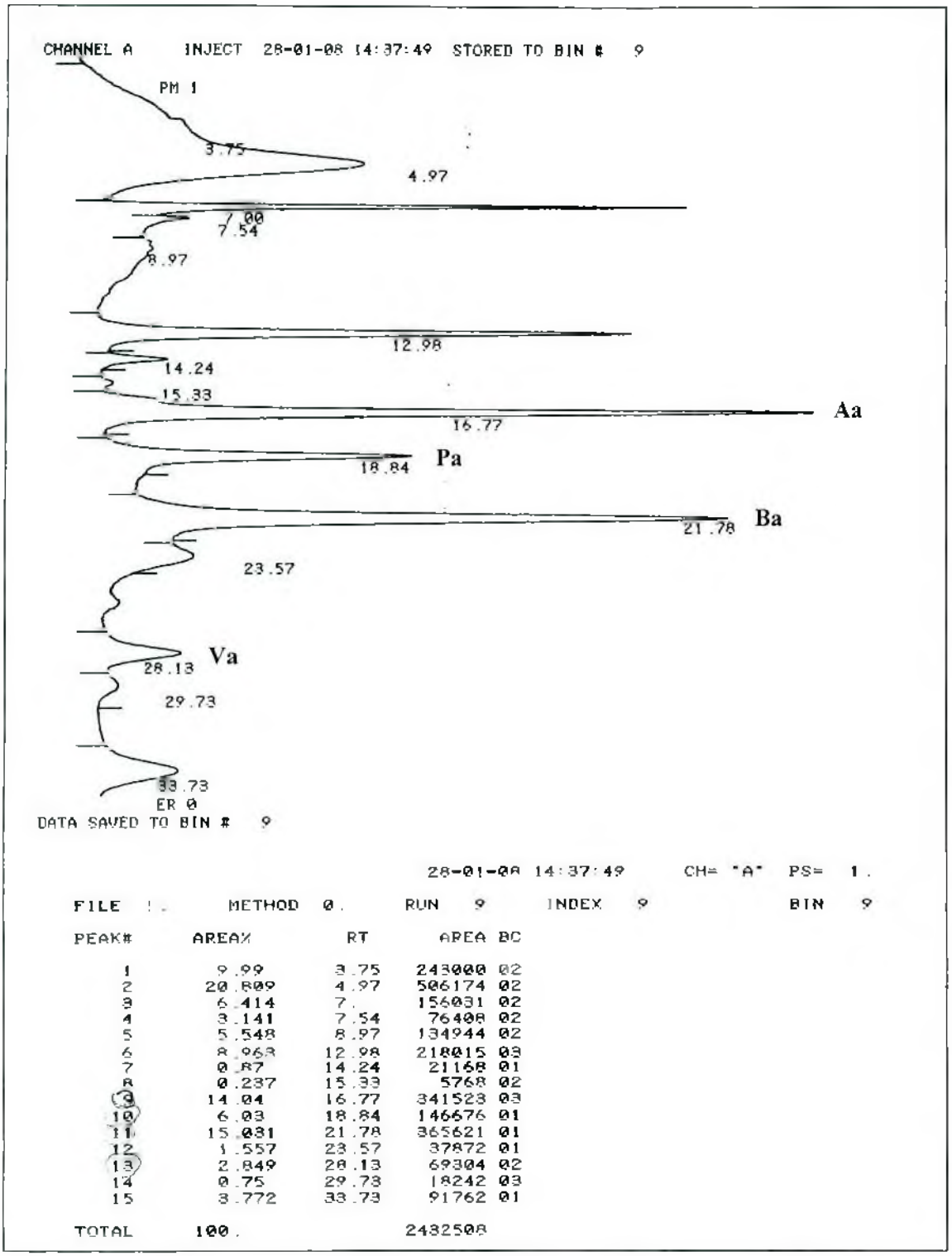
The concentration of short chain fatty acids in faeces e.g., acetic, propionic, butyric and valeric acids were analysed by HPLC. In acute cholera stage, the total concentration of these major fatty acids was only 4.7 ± 0.6 mmol/kg. The concentration began to increase significantly ($P < 0.01$) from day 1 until end of the study. At day 3 (during cessation of cholera), at day 7 (after completion of antibiotic), at day 28 (after one month of the cholera episode), the concentration was 30.9 ± 4.3, 51.7 ± 7.1 and 95.0 ± 8.7 mmol/kg, respectively. The concentration in healthy and malnourished children was 102.0 ± 14.4 and 70.6 ± 38.9 mmol/kg, respectively. Surprisingly, the concentration was not significantly different than the malnourished children with cholera at day 28. Table 20 shows the total concentration of acids in cholera children and control children.

Amongst different ORS treatment groups, no difference was found in concentration of SCFAs at day 0. At day 1, the concentration increased significantly in patients of rice-ORS group compared to glucose-ORS and glucose-ORS plus ARS group ($P = 0.007$ and 0.011 respectively). At day 2, the concentration increased significantly in patients treated with rice-ORS ($P = 0.007$) than glucose-ORS. The difference was significant between rice and glucose-ORS ($P = 0.047$) and between rice and glucose-ORS plus ARS ($P= 0.039$) at day 3. Afterwards, the concentration was increased steadily up to day 28 in all three treatment groups. No difference was observed at day 7 and at day 21 between the groups. At day 28, the concentration was only significantly lower in glucose ORS group than rice ORS group ($P = 0.029$). Data presented in Table 20. A typical chromatogram showing the different acids peak of a patient at day 21 was presented in Figure 33.

Table 20. Total Short Chain Fatty Acids in stool of study children.

Group	Malnourished children with cholera (N=30) (mmol/kg)							Mal. Ch. (N=11) (mmol/kg)	Heal. Ch. (N=6) (mmol/kg)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	4.4 ± 1.4	9.1 ± 2.2	9.8 ± 2.6	26.7 ± 5.8	43.2 ±10.8	74.3 ± 16.4	71.0 ± 8.6		
B (N=10)	5.1 ± 1.1	10.1 ± 1.9	16.8 ± 2.8	23.6 ± 5.7	42.4 ± 7.4	70.1 ± 9.3	95.5 ± 4.5		
C (N=10)	4.8 ± 0.8	22.8 ± 4.9	23.1 ± 3.9	47.1 ± 8.7	71.4 ±16.7	97.0 ± 8.9	113.3 ± 19.5		
Total	4.7 ± 0.6	14.0 ± 2.2	16.8 ± 2.1	30.9 ± 4.3	51.7 ± 7.1	80.9 ± 6.7	95.0 ± 8.7	70.6 ± 38.9	102.5 ± 14.4

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.



28-01-08 14:37:49 CH= "A" PS= 1.

FILE 1. METHOD 0. RUN 9 INDEX 9 BIN 9

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3	6.414	7.	156031	02
4	3.141	7.54	76408	02
5	5.548	8.97	134944	02
6	8.963	12.98	218015	03
7	0.87	14.24	21168	01
8	0.237	15.33	5768	02
9	14.04	16.77	341523	03
10	6.03	18.84	146676	01
11	15.031	21.78	365621	01
12	1.557	23.57	37872	01
13	2.849	28.13	69304	02
14	0.75	29.73	18242	03
15	3.772	33.73	91762	01
TOTAL	100.		2432508	

Figure 33. Chromatogram of faecal fatty acids of a patient at day 21 by High Performance Liquid Chromatography.

Aa, Acetic acid; Pa, Propionic acid; Ba, Butyric acid and Va, Valeric acid.

5.5.2 Concentration of individual acids produced in faecal specimen

Among different SCFAs, the concentration of acetic acid increased at significant quantity compared to other SCFAs and the concentration was noticed at day 1, followed by propionic, butyric and valeric acid on the following days. Detailed data was presented in Table 21, 22, 23 and 24.

Table 21. Amount of acetic acid in stool of study children.

Group	Malnourished children with cholera (N=30) (mmol/kg)							Mal. Ch. (N=11) (mmol/kg)	Heal. Ch. (N=6) (mmol/kg)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	2.9 ± 0.9	6.2 ± 1.8	7.0 ± 1.9	23.3 ± 5.9	25.2 ± 5.4	38.1 ± 9.2	38.7 ± 4.8		
B (N=10)	3.9 ± 0.8	8.7 ± 1.4	14.7 ± 2.4	16.9 ± 4.3	25.7 ± 3.4	41.7 ± 5.2	50.9 ± 3.9		
C (N=10)	3.5 ± 0.7	19.3 ± 4.8	19.1 ± 3.8	39.2 ± 8.5	38.8 ±13.1	49.0 ± 4.5	45.3 ± 6.8		
Total	3.5 ± 0.5	11.4 ± 2.0	13.8 ± 1.9	27.5 ± 4.3	29.6 ± 4.6	43.3 ± 3.5	45.0 ± 3.3	32.3 ±6.5	52.4 ±2.3

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

Table 22. Amount of propionic acid in stool of children.

Group	Malnourished children with cholera (N=30) (mmole/kg)							Mal. Ch. (N=11) (mmole/kg)	Heal. Ch. (N=6) (mmole/kg)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	1.0 ± 0.5	0.7 ± 0.6	0.5 ± 0.3	1.1 ± 0.5	14.9 ± 5.2	17.1 ± 4.2	14.6 ± 3.6		
B (N=10)	0.5 ± 0.2	0.5 ± 0.4	1.3 ± 0.7	5.3 ± 3.3	14.8 ± 5.0	15.6 ± 6.3	26.8 ± 4.8		
C (N=10)	0.4 ± 0.1	2.0 ± 1.3	2.0 ± 0.9	6.1 ± 2.2	24.5 ± 6.2	25.8 ± 5.6	35.7 ± 6.1		
Total	0.7 ± 0.2	1.2 ± 0.5	1.3 ± 0.4	4.0 ± 1.2	17.9 ± 3.2	19.7 ± 3.3	26.6 ± 3.4	19.1 ± 6.4	30.9 ±4.2

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

Table 23. Amount of butyric acid in stool of children.

Group	Malnourished children with cholera (N=30) (mmole/kg)							Mal. Ch. (N=11) (mmole/kg)	Heal. Ch. (N=6) (mmole/kg)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	0.4 ± 0.2	0.7 ± 0.5	1.2 ± 0.7	1.1 ± 0.5	2.5 ± 1.1	16.8 ± 5.2	13.7 ± 3.7		
B (N=10)	0.3 ± 0.1	0.3 ± 0.2	0.6 ± 0.3	0.8 ± 0.5	1.7 ± 0.5	11.9 ± 2.9	16.4 ±5.5		
C (N=10)	0.4 ± 0.1	0.6 ± 0.3	0.8 ± 0.4	1.5 ± 0.4	5.9 ± 1.6	18.8 ± 3.8	26.0 ± 8.9		
Total (N=30)	0.4 ± 0.1	0.5 ± 0.2	0.9 ± 0.3	1.2 ± 0.3	3.3 ± 0.7	15.8 ± 2.2	19.3 ± 4.0	15.4 ± 5.7	17.4 ±2.4

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

Table 24. Amount of valeric acid in stool of children.

Group	Malnourished children with cholera (N=30) (mmol/kg)							Mal. Ch. (N=11) (mmol/kg)	Heal. Ch. (N=6) (mmol/kg)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	0.03 ±0.01	1.5 ± 0.9	1.0 ± 0.8	1.5 ± 0.8	0.6 ± 0.4	2.4 ± 1.1	3.9 ± 1.5		
B (N=10)	0.4 ± 0.2	0.6 ± 0.3	0.2 ± 0.1	0.5 ± 0.3	0.3 ± 0.2	0.9 ± 0.5	1.4 ± 0.7		
C (N=10)	0.4 ± 0.2	0.9 ± 0.4	1.1 ± 0.4	0.3 ± 0.2	2.1 ± 1.2	3.5 ± 1.5	6.2 ± 2.3		
Total (N=30)	0.3 ± 0.1	1.0 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.9 ± 0.4	2.2 ± 0.6	4.1 ± 1.1	3.8 ± 1.4	1.7 ±0.5

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6 Analysis of faecal microbiota structure by fluorescent in situ hybridization (FISH) combined with Flow cytometry

5.6.1 Enumeration of bacterial cells

The number of bacteria (7.9 ± 0.08 log10/g, mean ± sem) was very low in acute cholera, day 0. The number increased with time and reached to 9.5 ± 0.03 log10/g at day 28. However, despite this increase at day 28 the bacterial number was lower in malnourished children with cholera and malnourished children without cholera (9.6 ± 0.08 log10/g) compared to healthy control group (10.0 ± 0.1 log10/g; $P<0.01$). In different ORS groups, the number of bacteria increased significantly faster in the rice-ORS group compared to glucose ORS ($P<0.01$) at day 1 and day 2. The bacterial number was intermediate in glucose ORS plus ARS group from day 1 to day 7 but the difference was not significantly higher than glucose or lower than rice at any time point. Detailed data were presented in Table 25.

Table 25. Number of Bacteria in stool of children.

Group	Malnourished children with cholera (N=30) (log10)/g							Mal. Ch. (N=11) (log10)/g	Heal. Ch. (N=6) (log10)/g
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	7.8 ±0.20	7.3 ±0.31	7.6 ±0.37	8.2 ±0.28	8.9 ±0.31	9.5 ± 0.13	9.6 ± 0.06		
B (N=10)	7.8 ±0.10	7.7 ±0.27	8.1 ±0.32	8.5 ±0.18	9.4 ±0.14	9.5 ± 0.15	9.4 ± 0.05		
C (N=10)	8.0 ± .13	8.4 ±0.18	8.8 ±0.12	8.7 ±0.21	9.3 ±0.16	9.4 ± 0.06	9.5 ± 0.06		
Total (N=30)	7.9 ±0.08	7.8 ±0.17	8.2 ±0.18	8.5 ±0.13	9.2 ± .12	9.5 ± 0.06	9.5 ± 0.03	9.63 ± 0.08	10.0 ± 0.1

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2 Detection of bacterial groups by probes targeted against 16S rRNA

5.6.2.1 Total bacteria

The distribution of different bacterial groups in stool specimens of patients treated with different ORS was further analysed by probes targeted against 16S rRNA. The sums of the bacterial group percentages were not different among the ORS treatment groups at day 0 and at the following time periods. On the other hand, sum of the bacterial group percentages in healthy children (87.7 ± 4.7) were significantly different ($P< 0.01$) than malnourished children with cholera at day 0 (40.4± 4.9), at day 28 (59.7± 4.8) and malnourished without cholera (52.5 ± 8.9). However, the total percentage of bacteria obtained by probes targeted against 16S rRNA at acute cholera phase was not different than malnourished children without cholera. Data was presented in Table 26.

Table 26. Proportion of faecal microbiota in study children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=6)	44.3 ±10.6	62.7 ±8.8	44.8 ±10.3	47.5 ± 9.8	56.0 ± 9.9	55.3 ± 6.8	61.4 ± 11.8		
B (N=8)	37.8 ± 6.9	55.7 ±11.8	62.8 ± 9.3	69.2 ± 8.7	62.3 ±10.0	53.0 ± 7.8	58.4 ± 9.5		
C (N=9)	39.4 ± 9.0	50.5 ±10.6	72.8 ± 6.9	53.9 ± 6.3	72.4 ± 5.8	55.3 ± 4.5	59.4 ± 4.2		
Total (N=30)	40.4 ± 4.9	56.5 ± 5.9	60.0 ± 5.4	56.9 ± 4.9	64.2 ± 4.9	54.5 ± 3.6	59.7 ± 4.8	52.5 ± 8.9	87.7 ±4.7

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2.2 The Enterobacteriaceae family (Proteobacteria phylum)

The average proportion of bacteria of Enterobacteriaceae family was higher (4.4 ± 0.9%) at day 0 in children with cholera than children without cholera and it increased approximately 10 times at day 1 in all three treatment groups. Although the proportion was lower in rice group at day 3, the difference was not significant between the groups. The proportion decreased gradually in the following days and reached 1.8 ± 0.8% at day 28. Healthy children had lower percentage of bacteria of Enterobacteriaceae family (0.81 ± 0.43%) than malnourished children (1.25 ± 0.4%). Table 27 presented the proportion of bacteria of Enterobacteriaceae family among the total microbiota in all the children.

Table 27. Incidence of Enterobacteriaceae family in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	4.5 ±1.83	46.2 ±11.3	22.26 ±11.2	24.8 ±11.8	7.7 ± 4.8	3.2 ± 3.1	0.42 ± 0.3		
B (N=10)	5.3 ± 2.1	47.5 ±12.3	37.6 ±11.8	32.8 ± 9.7	7.7 ± 3.7	1.0 ± 0.8	3.8 ± 2.3		
C (N=10)	3.3 ± 1.3	36.2 ±12.7	41.6 ±11.7	15.1 ± 5.3	19.4 ± 8.9	3.6 ± 1.5	1.1 ± 0.4		
Total (N=30)	4.4 ± 0.9	43.5 ±6.8	33.7 ±6.6	24.3 ± 5.4	12.8 ± 3.8	2.6 ± 1.0	1.8 ± 0.8	1.25 ±0.4	0.81 ±0.4

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**. Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2.3 The Clostridium coccoides group (Firmicutes phylum)

The members of *C. coccoides* group formed the average proportion of 10.7 ± 2.6% at day 0 among the total faecal microbiota in the children with cholera. It decreased to 1.6 ± 0.6% at day 1 and increased again to 8.9 ± 1.9% after 3 weeks of recovery. At day 3, the average proportion was higher in Glucose-ORS plus ARS than the two other treatment groups and the difference was significantly higher than rice group ($P < 0.05$, one way anova). There was significant difference of this bacterial proportion between malnourished children without cholera and healthy children, 4.8 ± 1.7% and 27.4 ± 3.7% respectively. Data presented in Table 28.

Table 28. Incidence of *C. coccoides* group in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	10.8 ± 5.6	2.7 ± 1.4	1.1 ± 0.6	0.8 ± 0.3	5.8 ± 3.7	11.53 ± 4.6	7.3 ± 3.53		
B (N=10)	9.5 ± 4.1	0.8 ± 0.3	0.8 ± 0.5	3.8 ± 1.8	4.2 ± 4.1	10.7 ± 2.9	4.9 ± 2.8		
C (N=10)	11.8 ± 4.2	1.2 ± 0.8	0.5 ± 0.1	0.4 ± 0.2	2.8 ± 1.3	10.8 ± 2.9	13.9 ± 2.7		
Total (N=30)	10.7 ± 2.6	1.6 ± 0.6	0.8 ± 0.3	1.7 ± 0.7	4.2 ± 1.8	10.9 ± 1.9	8.9 ± 1.9	4.8 ± 1.7	27.4 ± 3.7

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2.4 The Bacteroides group (Bacteroidetes phylum)

The members of Bacteroides group possessed lower proportion at day 0 than the malnourished children without cholera, 7.7 ± 2.1% and 10.7 ± 3.4%, respectively. The number decreased at day 1. At day 3, children of Glucose-ORS plus ARS and rice-ORS possessed higher proportion of this group of bacteria than Glucose-ORS but the difference was not significant. Healthy children possessed higher proportion of bacteroides (16.1± 4.0%) than malnourished children. Data presented in Table 29.

Table 29. Incidence of Bacteroides group in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	8.4 ± 3.5	2.2 ± 1.2	1.7 ± .9	1.8 ± .8	14.2 ± 5.9	2.8 ± 1.3	4.4 ± 3.5		
B (N=10)	7.9 ± 4.1	0.9 ± 0.4	3.3 ± 2.6	9.2 ± 5.6	5.2 ± 2.5	4.9 ± 2.7	3.4 ± 2.6		
C (N=10)	6.9 ± 3.4	1.8 ± 1.3	5.9 ± 3.1	9.3 ± 3.4	10.2 ± 3.5	5.9 ± 3.4	10.7 ± 5.4		
Total (N=30)	7.7 ± 2.1	1.6 ± 0.6	3.6 ± 1.4	6.8 ± 2.2	9.7 ± 2.3	4.7 ± 1.6	6.4 ± 2.4	10.7 ± 3.4	16.1 ± 4.0

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.5.2.5 The Faecalibacterium prausnitzii subgroup (Clostridium leptum group, Firmicutes phylum)

The average proportion of bacteria of *F. prausnitzi* subgroup in malnourished children with cholera was higher both at acute cholera (4.9 ± 1.2 %) and recovery stage (7.9 ± 2.2%) than the malnourished children without cholera. At day 3, the proportion was higher in children of rice ORS group than glucose-ORS and glucose-ORS plus ARS but the difference was not significant. Healthy children possessed similar proportion like cholera children after one month. Data presented in Table 30.

Table 30. Prevalence of *F. prausnitzi* subgroup in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	6.0 ± 2.3	0.7 ± 0.5	0.2 ± 0.1	0.4 ± 0.2	6.3 ± 4.6	7.3 ± 2.3	6.9 ± 4.2		
B (N=10)	3.5 ± 1.5	0.07 ± 0.06	0.9 ± 0.6	0.3 ± 0.2	2.5 ± 2.3	6.3 ± 2.3	7.4 ± 4.8		
C (N=10)	5.3 ± 2.5	3.1 ± 2.9	0.7 ± 0.3	2.0 ± 1.4	2.6 ± 1.3	6.3 ± 2.3	9.4 ± 2.9		
Total (N=30)	4.9 ± 1.2	1.2 ± 0.9	0.6 ± 0.2	0.9 ± 0.5	3.7 ± 1.6	6.6 ± 1.3	7.9 ± 2.2	3.2 ± 1.7	7.9 ± 2.8

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2.6 The Bifidobacterium genus (Actinobacteria phylum)

At day 0, the average proportion of bacteria of Bifidobacterium genus was lower in malnourished cholera children (6.4 ± 1.4%) than non-cholera malnourished children (23.2 ± 6.9%) and it decreased further at day 1 after antibiotic therapy. At day 3, the highest proportion was observed in children of rice-ORS (17.6 ± 9.0%), followed by glucose-ORS plus ARS (14.3 ± 7.2%) and glucose-ORS (3.6 ± 3.4%) group. The proportion was not higher in healthy children than malnourished children without cholera. Table 31 showed the detailed data of this bacterium in children faeces.

Table 31. Prevalence of Bifidobacterium genus. in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	5.9 ± 2.3	3.9 ± 1.9	2.1 ± .9	3.6 ± 3.4	4.2 ± 2.4	23.8 ± 7.5	32.6 ± 9.6		
B (N=10)	7.1 ± 2.1	2.9 ± 1.2	7.8 ± 3.9	14.3 ± 7.2	32.2 ± 10.5	19.3 ± 7.1	19.5 ± 7.0		
C (N=10)	6.2 ± 3.0	5.8 ± 5.0	7.3 ± 4.5	17.6 ± 9.0	18.0 ± 7.2	14.4 ± 3.4	9.4 ± 2.2		
Total (N=30)	6.4 ± 1.4	4.1 ± 1.7	5.7 ± 1.9	11.9 ± 4.0	18.7 ± 4.8	18.8 ± 3.5	19.9 ± 4.2	23.2 ± 6.9	18.1 ±5.6

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2.7 The Atopobium cluster (Actinobacteria phylum)

The members of Atopobium cluster was decreased by proportion in acute cholera state (3.8 ± 0.9 %) than non cholera malnourished state (6.8 ± 1.9%). After initiation of antibiotic therapy the bacterial percentage was reduced. At day 3, higher proportion of bacteria was observed in glucose-ORS plus ARS than rice and glucose-ORS but the difference was not significant. Malnourished children without cholera did not possess higher proportion than healthy children. Data presented in Table 32.

Table 32. Prevalence of Atopobium cluster in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	3.8 ± 1.3	0.9 ± 0.3	0.9 ± 0.4	0.3 ± 0.1	3.0 ± 2.3	4.6 ± 2.7	6.7 ± 3.9		
B (N=10)	2.9 ± 1.3	2.6 ± 2.1	6.4 ± 5.6	5.1 ± 3.2	5.8 ± 3.8	3.6 ± 1.2	12.1 ± 3.9		
C (N=10)	4.7 ±2.1	1.3 ± 0.6	0.9 ± 0.4	1.2 ± 0.6	9.4 ± 3.5	8.1 ± 2.5	7.5 ± 1.7		
Total (N=30)	3.8 ±0.92	1.6 ±0.76	2.6 ± 1.7	2.2 ± 1.1	6.3 ± 1.9	5.5 ± 1.3	8.7 ± 1.9	6.8 ± 1.9	10.2 ±1.5

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.6.2.8 The Lactobacillus-Enterococcus group (Firmicutes phylum)

The proportion of Lactobacillus-Enterococcus group of bacteria was different than other bacterial groups described above. It was higher in glucose-ORS group than rice-ORS and ARS-ORS group until day 7 started from day 0. At day 3, compared to ARS-ORS group, children of rice-ORS group possessed higher proportion of Lactobacillus-Enterococcus group of bacteria although the difference was not significant between any groups at day 3. Healthy children possessed higher proportion than malnourished children. Data presented in Table 33.

Table 33. Prevalence of *Lactobacillus-Enterococcus sp.* in faecal microbiota of children.

Group	Malnourished children with cholera (N=30) (%)							Mal. Ch. (N=11) (%)	Heal. Ch. (N=6) (%)
	Day0	Day1	Day2	Day3	Day7	Day21	Day28		
A (N=10)	4.9 ± 2.2	6.0 ± 4.8	16.5 ± 7.5	15.6 ± 7.9	14.8 ± 6.2	2.1 ± 1.0	3.1 ± 1.3		
B (N=10)	2.4 ± 0.6	0.9 ± 0.5	6.0 ± 3.7	3.4 ± 1.4	4.7 ± 1.2	7.1 ± 2.1	7.2 ± 2.8		
C (N=10)	1.9 ± 0.8	1.2 ± 0.4	15.8 ± 7.7	8.4 ± 2.7	10.0 ± 4.3	6.2 ± 1.8	7.3 ± 1.6		
Total (N=30)	2.9 ± 0.8	2.7 ±1.68	13.0 ± 3.8	9.1 ± 2.9	9.6 ± 2.5	5.4 ± 1.1	5.9 ± 1.2	2.5 ± 0.9	7.1 ±4.5

Values are expressed as mean ± sem. **A**, Glucose-ORS; **B**, Glucose-ORS plus ARS; **C**, Rice ORS. **Mal. Ch.**, Malnourished children without cholera; **Heal. Ch.**, Healthy children.

5.7 Impact of malnutrition, cholera and carbohydrate substrates of ORS on microbiota structure and SCFAs concentration in faeces of children

5.7.1 Malnutrition

The effect of malnutrition on faecal microbiota structure was analysed by comparing the variables between malnourished and healthy children (Figure 34). All the bacterial groups possessed higher proportion except Bifidobacterium in healthy children, but only *C. coccoides* group was significantly higher ($P<0.01$) in this group of children among the six anaerobic bacteria. The proportion of different bacterial groups in malnourished vs. healthy children was 1.25 ± 0.4% vs. 0.81 ± 0.4% for Enterobacteriaceae, 4.8 ± 1.7% vs. 27.4 ± 3.7% for *C.coccoides*, 10.7 ± 3.4% vs. 16.1 ± 4.0% for Bacteroides, 3.2 ± 1.7% vs. 7.9 ± 2.8% for *F. prausnitzii*, 23.2 ± 6.9% vs. 18.1 ± 5.6% for Bifidobacteria, 6.8 ± 1.9% vs. 10.2 ± 1.5% for Atopobium and 2.5 ± 0.9% vs. 7.1 ± 4.5% for Lactobacillus.

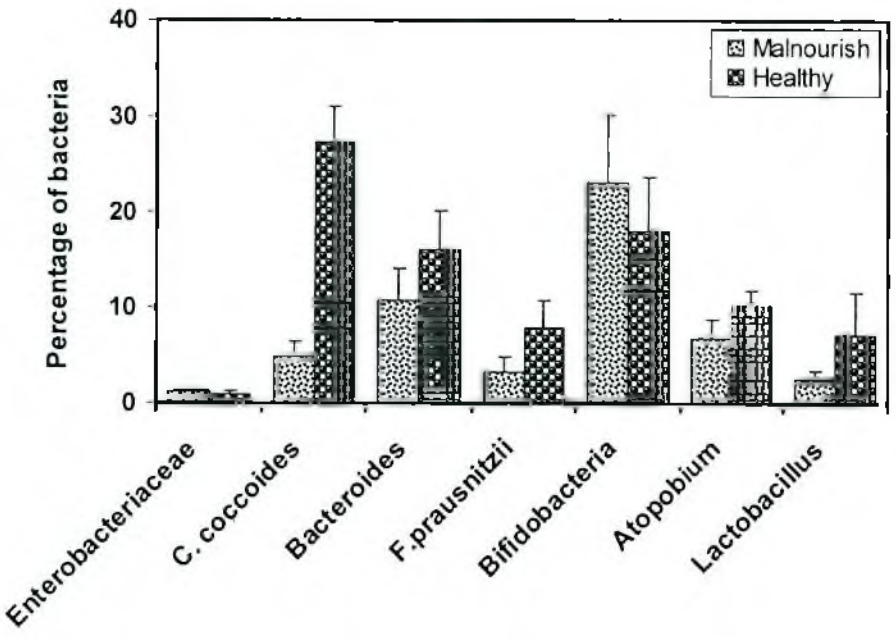

Figure 34. Effect of malnutrition on faecal microbiota composition in malnourished and healthy children.

Four different short chain fatty acids concentrations were compared between malnourished and healthy children to see the effect of malnutrition on faecal fatty acids concentration (Figure 35). It was observed from the analysis that, only acetic acid concentration was significantly higher (P=0.011) in healthy children. The total concentration of acids between malnourished vs. healthy children was 32.3 ± 6.5 vs. 52.4 ± 2.3 mmol/kg for acetic acid, 19.1 ± 6.4 vs. 30.9 ± 4.2 mmol/kg for propionic acid, 15.4 ± 5.7 vs. 17.4 ± 2.4 mmol/kg for butyric acid and 3.8 ± 1.4 vs. 1.7 ± 0.5 mmol/kg for valeric acid.

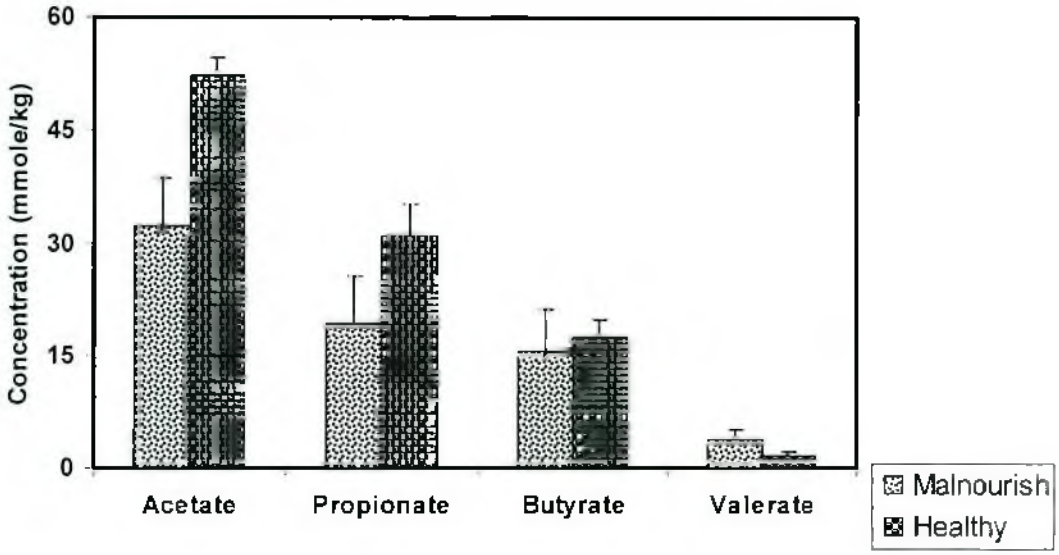

Figure 35. Effect of malnutrition on faecal short chain fatty acids concentration in malnourished and healthy children.

5.7.2 Cholera

The effect of cholera on faecal microbiota composition was analysed by comparing the proportion of different bacterial groups between severely malnourished children with cholera and without cholera (Figure 36). There was no significant reduction of anaerobic bacterial groups observed in acute cholera condition. Only Enterobacteriaceae was found significantly higher ($P<0.05$) and Bifidobacteria was significantly lower ($P<0.01$) in cholera children compared to non-cholera children. The proportion of different bacterial groups in cholera vs. malnourished status were as follows: 4.4 ± 0.9 vs. 1.25 ± 0.4% for Enterobacteriaceae, 10.7 ± 2.6 vs. 4.8 ± 1.7% for *C. coccoides*, 7.7 ± 2.1 vs. 10.7 ± 3.4% for Bacteroides, 4.9 ± 1.2 vs. 3.2 ± 1.7% for *F. prausnitzii*, 6.4 ± 1.4 vs. 23.2 ± 6.9% for Bifidobacteria, 3.8 ± 0.9 vs. 6.8 ± 1.9% for Atopobium and 2.9 ± 0.8 vs. 2.5 ± 0.9% for Lactobacillus.

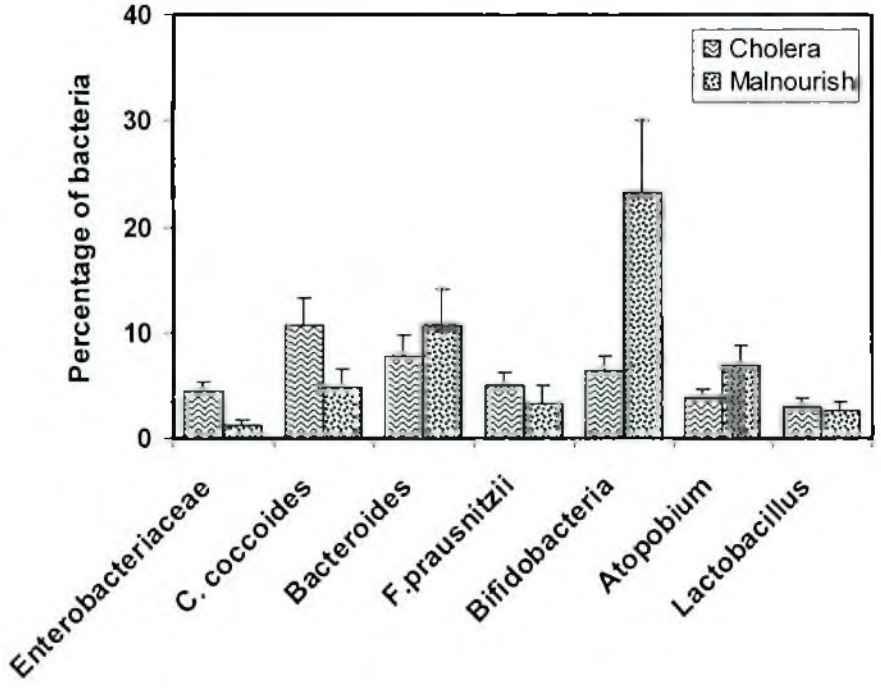

Figure 36. Effect of cholera on faecal microbiota composition structure in severely malnourished children with and without cholera.

Cholera has severe effect on short chain fatty acids concentration and each acid was found significantly lower in acute cholera condition ($P<0.01$, Figure 37). The concentration of different acids in severely malnourished children with cholera vs. without cholera (malnourished) condition were 3.5 ± 0.5 vs. 32.3 ± 6.5 mmol/kg in acetic acid, 0.7 ± 0.2 vs. 19.1 ± 6.4 mmol/kg in propionic acid, 0.4 ± 0.1 vs. 15.4 ± 5.7 mmol/kg in butyric acid and 0.3 ± 0.1 vs. 3.8 ± 1.4 mmol/kg in valeric acid respectively.

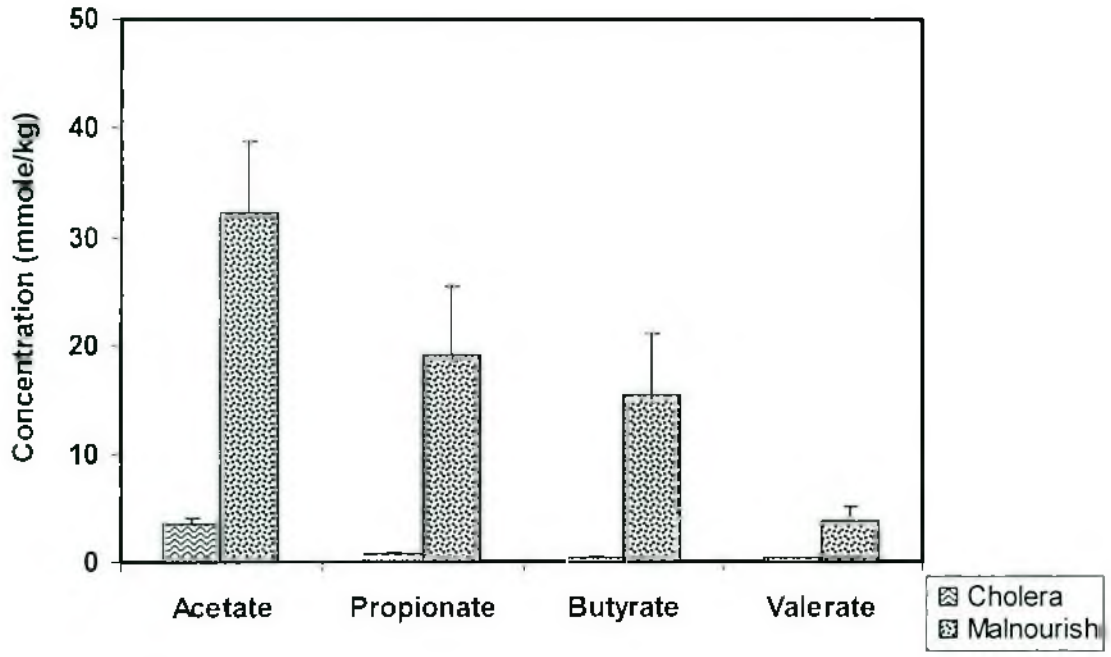

Figure 37. Effect of cholera on faecal short chain fatty acid concentration in severely malnourished children with and without cholera.

5.7.3 Impact of treatment with oral rehydration solution containing different carbohydrate substrates on faecal microbiota structure and fatty acid concentration

During recovery from cholera, the effect of oral rehydration solution containing different carbohydrate substrates on faecal microbiota composition was analysed by comparing the percentage of each bacterial group at day 3 between three treatment groups of severely malnourished children with cholera (Figure 38). It was observed that, among the six anaerobic bacteria there was a trend of clustering higher proportion of Lactobacillus-Enterococcus in glucose-ORS group, *C. coccoides* and Atopobium in glucose-ORS plus ARS group and *F. prausnitzii* and Bifidobacterium in rice-ORS group. Bacteroides proportion was higher and similar in both rice and ARS-ORS group than glucose-ORS. No significant difference was observed between treatment groups for any bacterial lineage except *C. coccoides* which was significantly higher in ARS-ORS group than rice. Sum of the bacterial groups was contributed mainly by Enterobacteriaceae. The proportion of different bacterial groups in glucose vs. ARS vs. rice group were as follows: 24.8 ± 11.8% vs. 32.8 ± 9.7% vs. 15.1 ± 5.3% for Enterobacteriaceae, 0.8 ± 0.3% vs. 3.8 ± 1.8% vs.0.4 ± 0.2% for *C. coccoides*, 1.8 ± 0.8% vs. 9.2 ± 5.6% vs. 9.3 ± 3.4% for Bacteroides, 0.4 ± 0.2% vs. 0.3 ± 0.2% vs. 2.0 ± 1.4% for *F. prausnitzii*, 3.6 ±3.4% vs. 14.3 ± 7.2% vs. 17.6 ± 9.0% for Bifidobacterium, 0.3 ± 0.1% vs. 5.1 ± 3.2% vs. 1.2 ± 0.6% for Atopobium, 15.6 ± 7.9 % vs. 3.4 ± 1.4% vs. 8.4 ± 2.7% for Lactobacillus-Enterococcus and for total proportion 47.5 ± 9.8 vs. 69.2 ± 8.7% vs. 53.9 ± 6.3% respectively.

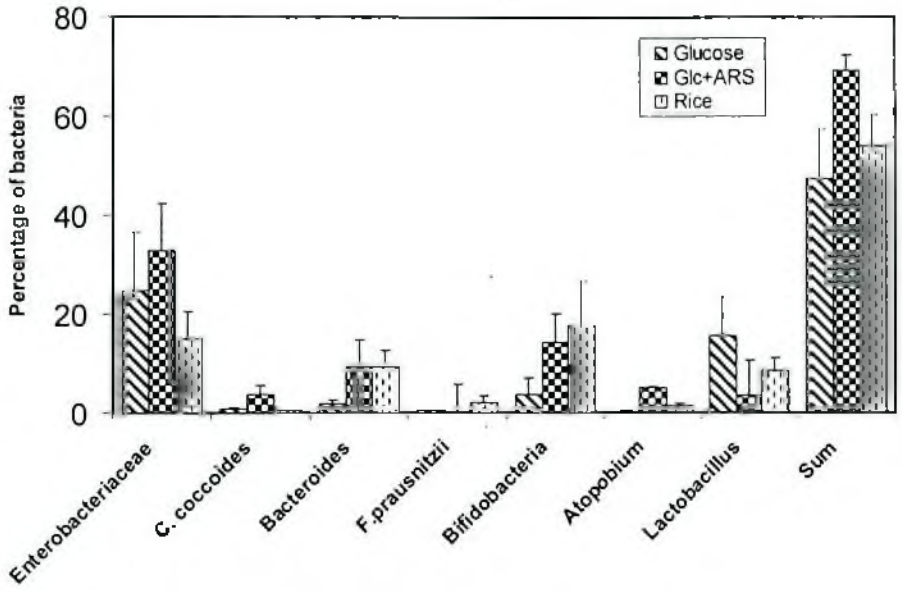

Figure 38. Effect of different carbohydrates on faecal microbiota structure in severely malnourished children with cholera.

The concentration of acetic acid was higher among the four different acids on day three and its concentration was significantly higher in rice-ORS than glucose-ORS-ARS group (Figure 39). Propionic acid concentration was observed next and higher concentration was found in ARS and rice ORS than glucose-ORS group. Total concentration was contributed mainly by acetic acid concentration. The concentration of different acids in glucose vs. ARS vs. rice group were 23.3 ± 5.9 vs. 16.9 ± 4.3 vs. 39.2 ± 8.5 mmol/kg for acetic acid, 1.1 ± 0.5 vs. 5.3 ± 3.3 vs. 6.1 ± 2.2 mmol/kg for propionic acid, 1.1 ± 0.5 vs. 0.8 ± 0.5 vs. 1.5 ± 0.4 mmol/ kg for butyric acid, 1.5 ± 0.8 vs. 0.5 ± 0.3 vs. 0.3 ± 0.2 mmol/kg for valeric acid and for total fatty acids 26.7 ± 5.8 vs. 23.6 ± 5.7 vs. 47.1 vs. 8.7 mmol/kg.

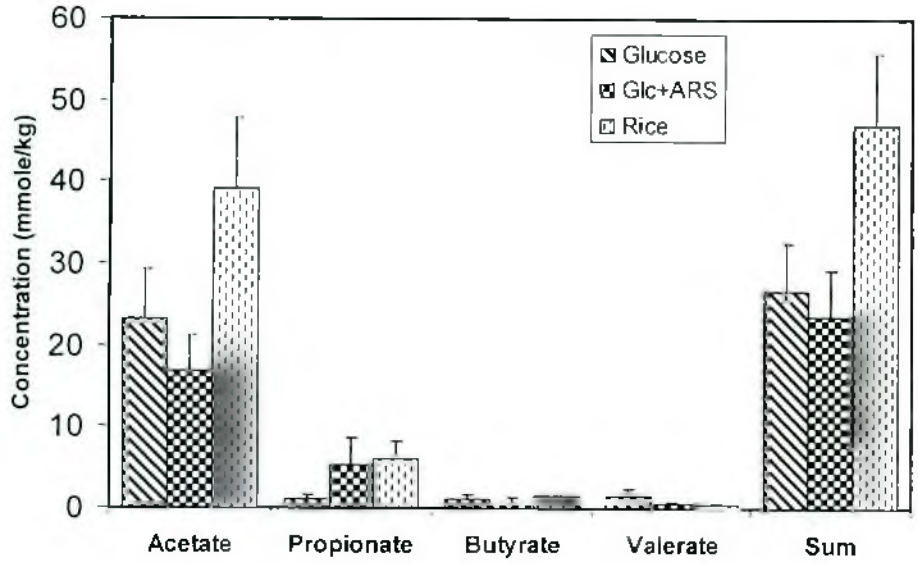

Figure 39. Effect of different carbohydrates on faecal short chain fatty acids concentration in severely malnourished children with cholera. Day 3 samples were analysed.

5.8 Evolution of microbiota and increasing of SCFAs during convalescence and nutritional period after cholera.

The level of some strictly anaerobic and facultatively anaerobic bacteria decreased during acute cholera stage and decreased further upon initiation of antibiotics. In course of time, the bacterial proportion increased again during convalescence and nutritional phase. Data analysed from all cholera children (N = 30) at seven different time periods and compared with malnourished children without cholera (N = 11) were presented in Figure 40. The main feature of this Figure was that the proportion of Enterobacteriaceae, the facultative anaerobic bacterial population of faecal microbiota was only 10% during acute cholera i.e., at day 0, which covered approximately 80% of faecal bacterial population at day 1. This proportion decreased gradually and came to the level of non-cholera children at day 28.

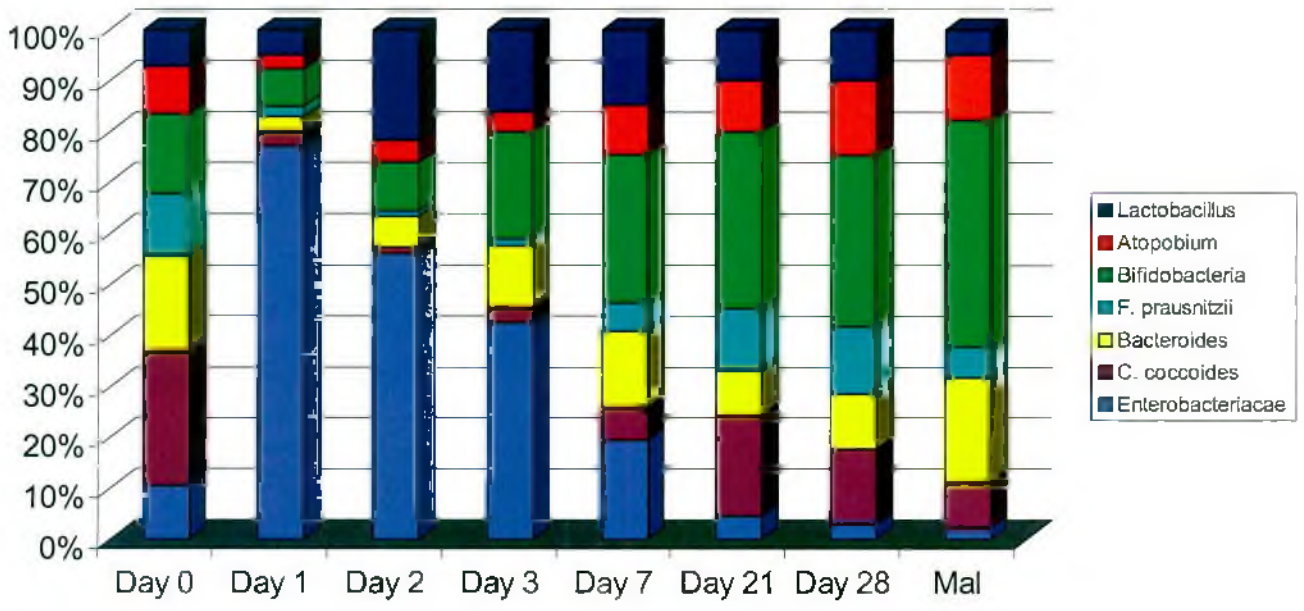

Figure 40. Evolution of faecal microbiota followed by acute cholera in study children. Mal, Severely malnourished children without cholera.

Short chain fatty acid (s) concentration was very low in acute cholera stage and started to increase from day 1 until end of the study. The increase in different SCFAs was significantly

different. The concentration of different SCFAs at different time points was analysed among all the severely malnourished children with cholera (N = 30) and presented in Figure 41.

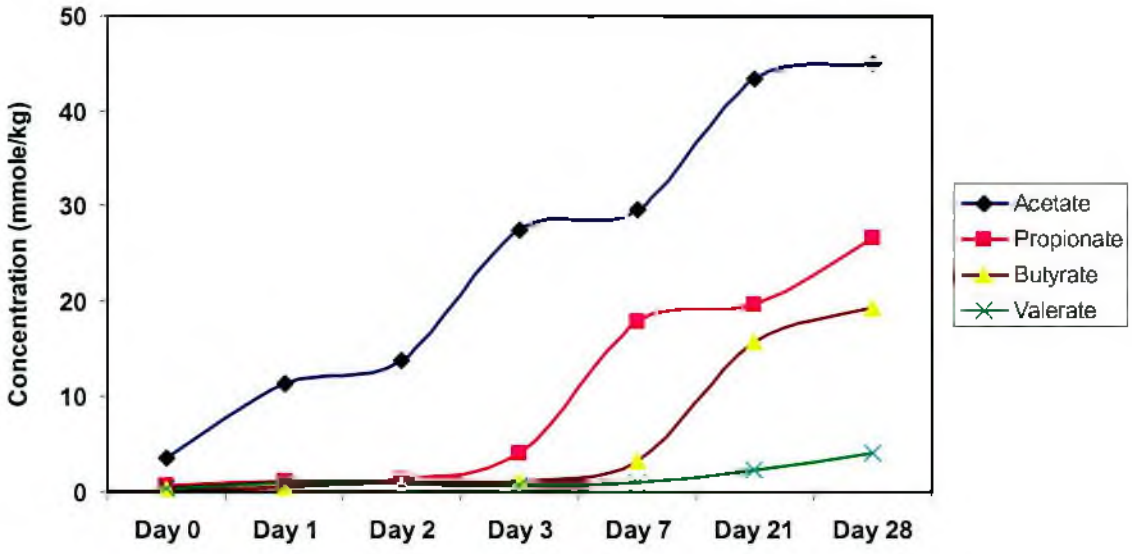

Figure 41. Increasing of short chain fatty acid concentration followed by acute cholera.

Chapter VI

Discussion

6.0 Discussion

The results of our study demonstrated transient alterations in the faecal bacterial diversities during acute cholera in severely malnourished children. The results also demonstrated that these children possessed considerably lower magnitude of diversity in their gut compared to healthy children. Bacterial diversity was increased significantly at day 3, in severely malnourished children with cholera during treatment with ORS containing complex carbohydrates. We observed that acute cholera caused reduction of bacterial number and the proportion of major components of faecal microbial community. The results indicated that cholera has a drastic effect on the concentration of short chain fatty acids, the major metabolic product of the colonic microbiota. During recovery bacterial number, proportion and concentration of SCFAs increased. While comparing between three ORS treatment groups, better effect was observed in children receiving Rice-ORS.

Improved colonic salvation of water and electrolytes in diarrheal patients and improved colonic function in nutritional interventions are topics of recent interest. The assumption is based on the fermentation of amylase-resistant carbohydrates by colonic bacteria liberating SCFAs that serves as metabolic fuel as well as a potent stimulator of water and electrolyte absorption (5, 23, 53, 98, 158, 164). As it is documented previously that small intestine plays the major role for rehydration during diarrhea through glucose based electrolyte and water absorption, the present study aimed at colonic absorption of salt and water through the stimulation of colonic bacteria and their metabolism. In addition to small intestinal absorption, the study has taken an attempt to evaluate the efficacy of two different oral rehydration solutions e.g., glucose-ORS supplemented with amylase resistant starch and cooked rice-ORS for rehydration of severely malnourished children with cholera while maintaining the glucose-ORS as the standard ORS for comparison. So, colonic bacterial population is the central point to utilize the starch reached the colon. However little is known about the status of the colonic flora in pathological conditions, particularly in cholera, the most severe form of diarrhea. Research investigation of such microbial habitat during cholera is important since it would give an opportunity to perform an in-depth study of the alteration of bacterial diversity, their proportion and their major metabolic product, the SCFAs as well as their restoration in the following days. At the same time this investigation would give a

privilege to measure what extent the diversity is influenced in the same microbial habitat by cholera and nutrition by comparing the malnourished cholera condition with non-cholera malnourished and healthy condition.

We studied for the first time, the faecal bacterial diversity, their number, proportion of key commensal bacteria and concentration of SCFAs in acute, convalescent and nutritional rehabilitation period of severely malnourished children with cholera. Previous studies have examined the gastrointestinal flora in acute cholera (82, 83) and in acute diarrhea (8, 81, 203) using conventional culture techniques, microscopy and biochemical tests inferred that the faecal anaerobic bacteria were significantly reduced in number during acute disease leading to aerobic bacteria to increase several fold. The major anaerobic bacterial group e.g., Bacteroides, Clostridium, Bifidobacterium, Lactobacillus and Eubacterium were found 3 to 4 fold lower in acute cholera and diarrhea.

Cultivation of anaerobic bacteria are difficult and requires the use of special apparatus such as anaerobic glove boxes, or test tubes flushed with an oxygen-free gas, to provide a suitably reduced environment that will enable extremely oxygen-sensitive bacteria to grow. Highly selective medium are also needed to culture them. The major handicap to successful analysis of the gut microflora has been the inability of bacteriologists to cultivate all of the members of the bacterial community under laboratory conditions. It is evidenced that about 60% of the total microscopic count cannot be cultured even with the best anaerobic culture methods (197). This problem has been solved to some extent in microbial ecology by the use of analytical approaches that are DNA-based and are referred to as molecular methods. The target within the bacterial chromosome for these DNA-based methods is usually the 16S rRNA gene (201). Molecular techniques e.g., direct cloning and sequencing of 16S RNA gene from a complex community DNA, dot-blot hybridization of rRNA or fluorescent in situ hybridization of whole cell followed by detection with DNA probes targeted against 16S rRNA, community finger print techniques such as denaturing gradient gel electrophoresis (DGGE) or temporal temperature gradient gel electrophoresis (TTGE) or T-RFLP and real time PCR of the 16S rDNA to estimate the bacterial load in a mixed community are the leading methods used by the environment microbiologists throughout the last two decades. These molecular techniques were used in determining genetic diversities among natural

microbial communities such as biofilms (136), soils (70), ocean depths (204), hot springs (72), fermented foods (39), human colonic biota (217), termites' gut (149) etc. The microbial community of human gut has drawn much attention in recent years as it came to know that, gut microbiota has profound relation with health and diseases. Recently, gut bacteria was investigated using molecular techniques in several different settings like disease conditions e.g., diarrhea (16), Crohn's disease (210), type 2 diabetes in children (ref); colonization of bacteria in different periods of age e.g., weaning period or gut flora in preterm baby (114, 116); intervention of prebiotic approach in babies (115); genetic variation and similarity in twin children (195) etc. However, information about gut microflora in acute cholera and recovery period by molecular techniques in severely malnourished children is lacking.

In view of the above facts, In the present study we used a culture independent method called TTGE to assess the faecal microbiota diversity comprising both aerobic and anaerobic bacteria in severely malnourished children with cholera and to examine the population shifts over a period of time (from day 0 to day 28).

Until now, correlation between host responses to intestinal infections and other external factors such as diet or antibiotics and changes in the intestinal bacterial populations has been difficult. In the present study, we collected stool samples from malnourished children with severe cholera to investigate bacterial diversities in their stools using culture independent newer techniques. During the acute phase of infection, we observed prominence of bands of *V. cholerae*; and in addition to that, the presence of fewer bands representing other bacterial species. This is in agreement with culture based studies in which investigators have observed reductions in bacterial species in watery stools of severely purging adult patients with cholera due to *V. cholerae* (40, 83). In this study, the use of the 16S rDNA based PCR-TTGE techniques demonstrated re-establishment of faecal bacterial community after an insult caused by cholera in severely malnourished children. However, faecal flora represents that in the terminal colon and does neither represent mucosal flora (which differ from those in the luminal) nor those in the proximal large gut.

In our study, severely malnourished children with cholera received appropriate rehydration and antimicrobial therapy, and continued feeding in accordance with the World Health Organization guidelines. Although antimicrobials exert a beneficial effect by controlling infection i.e. by eliminating pathogenic agents, administration of oral agents often causes a shift in the intestinal microbial populations, with a decrease in anaerobic populations and a concomitant increase in the aerobic populations (18, 179). In this study, the absence of antibiotic-specific differences in band numbers suggested that microbial diversity, as reflected by the number of different TTGE bands did not significantly alter at day 1 i.e. 24 hours after initiation of therapy. On the other hand, analysis of distance values between day 0 and day 1 showed significant changes in the bacterial species. Thus the present results suggest that antibiotic therapy maintained the diversity combined with decreased similarity between bacterial profile of day 0 and day 1. Such an alteration was transient.

We observed substantial inter-individual variations in the number of TTGE bands (4 to 17) among severely malnourished children with cholera from day 0 to the end of the study period. These children were randomly selected from those attending the outdoor of the Dhaka Hospital on the basis of inclusion criteria for the main study. They came from different localities and were on different diets. As diet and metabolism have profound relation with indigenous bacterial population (32, 75), it is possible that these children possessed individual band profiles related to their diets. However, during their hospitalization (recovery period), they received similar volume of rehydration solution followed by similar types of semi-solid and solid foods, yet the increase in band numbers did neither follow the similar pattern nor similar rates, which tends to suggest that changes in colonic flora might differ between individuals.

All study children were observed in the special, designated Research unit of the hospital until resolution of cholera has occurred and to attain their 80% weight for height took around seven days. Day 21 and day 28 samples were collected from their homes. Some distinct and very dominant bands were observed in all TTGE profiles until day 7, although the position of these bands was not consistent in every patient. On the other hand, higher number of bands was found on day 21 and 28, compared to day 7, whereas no bands were found in these samples with higher intensities. One possible reason for this observation could be that fewer

bacterial species could have proliferated in presence of antibiotics during the treatment period, and some others were suppressed at the same time. This is similar to the observations that administration of ampicillin to humans often results in the production of β-lactamase by various intestinal microorganisms, including *Enterobacteriaceae*, and some members of the *Bacteroides fragilis* group, with concomitant multiplication of ampicillin-susceptible *Clostridium difficile* (120, 166).

A remarkable difference in species richness (the number of different species), as characterized by the TTGE band numbers, was observed when cholera children's' TTGE profiles was compared with the healthy children. We observed 20 to 24 bands in healthy children compared to only 9 to 17 bands in cholera children, at day 28. This indicates that healthy children possessed microbiota of greater diversity in their colon compared to malnourished children with or without cholera. Our observed number of bands was similar to that was observed in an earlier study (195) using TTGE, which analyzed influence of host genetics on the predominant bacteria in the faecal microbiota of identical twins, fraternal twins and unrelated pairs, age between 4 months and 10 years- the number of bands in TTGE varied from 8 to 28.

The simplicity of the gut bacterial community in malnourished children may be due to their compromised nutritional status, narrower range of foods they received, poor absorption of nutrients and metabolism. One of the main functions played by the indigenous microbiota in large intestine is colonization competition, in which the intrinsic bacteria try to prevent colonization of extrinsic bacteria, which could be pathogenic. In the present study as the malnourished children harbored a relatively less diverse resident microflora compared to healthy children, they are more prone to colonization by foreign bacteria and to recurrent illness (120, 190).

The intestinal microbiota degrades and ferments dietary components that escape digestion in the small gut and endogenous substances that were produced along the intestinal tract. These dietary components are mainly carbohydrates (resistant starches, fibres and non-digestible oligosaccharides), proteins and mucins. The degradation and fermentation of these substrates in the colon requires the contribution of different groups of microbiota linked in a trophic

chain transforming dietary polysaccharides into smaller fragments that are further fermented into short chain fatty acids represented by acetate, propionate and butyrate and gases, mainly H_2 and CO_2 (103).

As expected in a severe episode of watery diarrhea, bacteria and short chain fatty acids concentrations were very low in the stools of children with acute watery diarrhea (16, 158). Then the concentrations of bacteria and short chain fatty acids increased with time. Here we also showed that the clinical recovery with time was associated with a typical pattern of bacteria and short chain fatty acids in stools: clinical rehydration was associated with a striking increase in both bacteria and SCFA concentrations. The increase was observed with all 3 carbohydrates in ORS, and better results were observed in the children receiving rice-ORS. In addition, normal nutrition was associated with further increase in bacteria and SFCA concentration.

The results appear to support the proposition to stimulate colonic SCFA production with rice-ORS in order to improve rehydration and nutrition in patients with severe malnutrition and cholera (22, 53, 55, 163, 164). However, the number of bacteria and SCFA in stools are only proxy markers of the metabolic activity in the different parts of the colon. For example, it is well established that SCFA produced in the colon are essentially absorbed locally (181, 205). Nevertheless, the low concentrations of bacteria and SCFA observed in the acute phase of high stool output are likely to represent the colonic status. It is unlikely that such a low stool concentration of bacteria and SCFA reflected a normal concentration in the colon. The repeated measurements during rehydration and nutrition periods on the same children for 28 days reflected the changes with time. As the children recovered from dehydration and malnutrition, the absorptive colonic function was likely to improve rather than deteriorate. Thus the increased SCFA concentration in stools may in fact under-estimate rather than over-estimate the increased SCFA production with time.

The total fatty acid concentration was found to be reduced drastically in acute cholera phase (4.7 ± 0.6 mmol/kg) irrespective of treatment groups and increased gradually up to 95.0 ± 8.7 mmol/kg at day 28. Among the individual acids, acetic acid concentration was increased first followed by propionic and butyric acid. The abundance of acetic acid is higher than other

acids. Butyric acid was noticeable only on day 3. The trend was similar to the work previously reported by Ramakrishna *et al.* in which they studied adult (age range 18 to 65yrs) with cholera, but the concentration of total fatty acids was not the same, they observed 9.9 ± 5.8 mmol/kg in acute cholera and 94.8 ± 16.4 mmol/kg at day 4 (156). We observed the higher concentration in severely malnourished children with cholera at day 28. Also we didn't expect similar concentration of SCFAs in adult and children.

In another work Ramakrishna *et al.* (adult with cholera) reported that, at day 2 the total acid concentration was 16.2 ± 11.8 mEq and 8.0 ±10.2 mEq of SCFAs in amylase resistant starch and glucose-ORS group, respectively which correlated the result of our study presented in Table 20. But the total acids concentration in Rice-ORS group did not correlate with the present study. They observed 8.0 ± 8.0 mEq, while the present study observed 23.1 ± 3.9 mmol (mEq and mmol are the similar units). The only difference was in rice ORS preparation and it was not cooked in that study (158).

From the clinical point of view, rehydration and improved nutritional status were associated with increased concentrations of bacteria and SCFA in stools, suggesting a causal relationship between both events. That hypothesis is supported by the observation that rehydration and stool output reduction was greater in children receiving rice-ORS solution, while faecal bacteria and SCFA concentrations also increased faster in this group from day 1 and onwards. The ability of SCFA to stimulate sodium and water absorption from the mammalian colon has been shown previously *in vivo* and *in vitro* (20, 34, 211). With amylase-resistant starch, we observed an intermediate clinical effect not only in the 30 studied children but also in the parent study of 170 patients (7). Again, with amylase-resistant starch, the concentrations of bacteria and SCFA were intermediate between rice and glucose groups. More intriguing are the results with glucose because glucose is supposed to be entirely absorbed in the small intestine. From the data it seems that, part of the glucose reached the colon or that endogenous carbohydrate metabolism improved with the status of the children, or both.

After rehydration, during the nutrition phase, the concentrations of both bacteria and SCFAs increased; it is an indication that the metabolic function of the colonic bacteria was transiently altered in the acute cholera phase. Full recovery to healthy level was not achieved even after 28 days. Although SCFAs remained higher in the group that received rice, we

could not identify a clinical benefit on nutritional recovery. It may be that the weight for height index was not the best marker to assess a nutritional benefit (71). It may also suggest that the statistical difference did not carry a significant metabolic effect. In that line, it must be emphasized that all the 30 children recovered; even in the large parent study, all the 170 severely malnourished children with cholera recovered whatever the differences in recovery rate at the rehydration phase (7). Clearly, in such a life threatening condition, survival is more important than stool output reduction, even if both events are interlinked.

We observed higher concentration of short chain fatty acid production in Rice-ORS group and the possible reasons may be laid in the characteristics of available starch in colon and the type of the commensal microbiota involved in fermentation of the starch. Normal native starches consist of a mixture of 15-30% amylose and 70-85% amylopectin. Amylose structurally is a linear polymer of anhydroglucose units (α-1, 4 linkage), of molecular weight is approximately between 40 000 and 340 000 dalton, the chains containing 250 to 2000 anhydroglucose units. Amylopectin is considered to be composed of anhydroglucose chains (α-1, 4 linkage) with many branch points (α-1, 6 linkage); the molecular weight may reach as high as 80 000 000 dalton. The amylose content of normal high yielding varieties of rice ranged from 15 to 22% of the total starch (159). Increasing the proportion of amylose in starch (rice, maize, barley) causes the resistant character of the starch to be increased to digestive amylases (214). The high-amylose maize starch which were supplied by Starch Australasia, Ltd., contained more than 80% amylose and 33.4% total dietary fiber (214). On the other hand, no amylose fraction was detected by amperometric titration with KIO_3 at low pH in normal rice(amylose:amylopectin ratio is 20:80) after cooking, assumed that the amylose was turned to oligosaccharides and glucose (207). So, the characteristics of starch of rice ORS in this study was more suitable for utilization in colon of severely malnourished children with cholera than the Hi amylose maize starch where high purging rate causes the partial washout of the commensal microbiota and changes of the strictly anaerobic environment to some extent aerobic.

The clinical benefit of rice-based ORS was probably due to several factores: hypo-osmolarity, which would enhance water absorption in small intestine, the potential to release more glucose than WHO-ORS and yielding of amino acids and oligopeptides from hydrolysis

of proteins of rice powder by intra-luminal enzymes which enhance sodium absorption through an independent carrier system (117, 130, 183), boiled rice powder possessing some anti-secretory factors that inhibits secretion of fluid from the small intestine (113) and SCFAs which stimulate sodium and water absorption through the colon (20, 21). From the clinical data of our study we observed that, the cholera children of rice-ORS group had significantly reduced stool output than glucose-ORS group and it was 30 to 40% during day 1 to day 3. We also observed that, the increase in total SCFAs concentration during day 1, day 2 and on day 3 were 151%, 136% and 76% respectively in contrast to glucose-ORS group. Some may argue that the concentration of SCFAs in stool of patients receiving rice-ORS might be due to the reduction in stool volume in rice-ORS group. However, the percentage increased in SCFAs concentration has much exceeded the percentage reduction in stool volume, which might explain some additive effect of SCFAs on water and sodium absorption through colon leading to stool volume reduction. As expected the similar result was not achieved in Glucose-ORS plus ARS group.

The SCFAs pattern was close to what could be expected from the bacterial pattern. The commensal microbiota (except Enterobacteriaceae) analyzed here are strictly anaerobic and each component of human gut takes part in fermentation of dietary components reached the colon. They are linked with each other through cross feeding of their metabolic products. Here the results suggest that the bacterial diversity may explain the SCFAs changing pattern with time. The pattern of the 3 main SCFAs, i.e. acetate, propionate and butyrate was might be due to presence of of higher proportion of the *Bacteroides sp.* and *Bifidobacteria sp.* in glucose-ORS plus ARS and in rice-ORS group and similarly from *Lactobacillus sp.* in glucose-ORS group. It is evidenced from some recent studies that *Bacteroides sp.* and *Bifidobacteria sp.* can stay concurrently. Biofilm analysis of human gut by confocal laser scanning microscopy (CLSM) showed that bacterial populations strongly adhering to particulate matter predominating with *Bacteroides sp.* and *Bifidobacteria sp.* Acetate was the principal fermentation product formed by biofilm bacteria, whereas higher levels of butyrate were produced by nonadherent populations, showing that the two communities were metabolically distinct (112). In another study with gnotobiotic mouse model showed that, *Bacteroides sp.* acquired an expanded level of capacity to degrade diverse polysaccharides when cooperated by *Bifidobacterium* and *Lactobacillus* (191). Again, in an invitro coculture fermentation study it has been showed that, *Bifidobacterium sp.* preferentially degrade shorter length of oligosaccharides to acetate, lactate, formate, ethanol and some extent succinate (69).

This characteristic of *Bifidobacterium sp.* correlate with its higher average proportion in the patients of rice ORS group where shorter length of oligosaccharide was available in greater extent.

We observed the higher proportion of *Lactobacillus sp.* in Glucose-ORS group in convalescent period although the reason was not clear. One possible reason may be that, these children received higher amount of breast milk from their mother than the other groups of children. *Lactobacillus sp.* mainly produces lactic acid by fermentation of available starch in colon but lactic acid is usually found in very low concentration in faecal samples. The reason is that some other members of the anaerobic community e.g., *Clostridium sp.*, *Megasphera sp.* immediately utilize lactic acid and turned it into acetic acid, propionic acid, and CO_2 (85). In another study it was found that some other components of this community e.g., *Eubacterium hallii* or *Anaerostipes caccae*, or some new species of Clostridium cluster XIVa utilized lactic acid to produce butyric acid (60). The proportion of this bacteria was lower on day 1, then increased on the following days until day 7 i.e., convalescent period and reduced again on day 21 and 28 i.e, nutritional period. The similar phenomenon was observed in a study by Balamurugan *et al.* in patients with diarrhea (16). *F. prausnitzii*, a common but minor component of faecal microbiota, was found to have a trend of having higher proportion in convalescence and nutritional period in children of Rice-ORS group. This component is a potent acetate consumer and participate actively in butyrate formation (59).

In our study we observed that, in acute cholera condition the severely malnourished children possessed moderate proportion of faecal anaerobic bacteria which was drastically reduced on day 1 (Figure 40). These children received doses of 3 antibiotics daily as their treatment guideline. The possible reason for this reduction may be that, antibiotics had a negative impact on the faecal anaerobic population and as a result these bacteria were eclipsed severely (199). On the following days when the purging rate was reduced and the anaerobic environment was restored in colon, these bacteria could proliferate and increased their proportion.

The study represented a striking correlation between total number of bacteria and the total concentration of SCFAs on day 3 compared to day 0 among all children with cholera. The bacterial number increased 0.6 log on day 3 than day 0 and the concentration of total fatty acids increased six fold on day 3.

The data showed that microbial proportion of *Enterobacteriaceae* group increased approximately 10 fold on day 1 (Table 27). Probably the suddenly increased population was resistant to antibiotics and increased sharply after initiation of it, although the reason was not clear. The similar instances could be found in some studies with antibiotics targeting effect on gut flora. Recently, Van Loon *et al.* reported that in clinical practice, quarterly homogeneous antibiotic exposure alternating quinolones and b-lactams increased Gram-negative microorganism resistance to the antibiotic in use (209). In some other instances, increased third generation cephalosporin use was associated with a significant increase in *Enterobacteriaceae* isolates including ESBL (extended-spectrum-beta lactamase) producing strains (178).

Although PCR-TTGE provided a suitable tool for evaluation of entire bacterial population of children with cholera at different time points and permitted analyses of a large number of samples, it is possible that minor species remained undetected by this technique. Additionally, the apparent community diversity may be decreased using PCR-TTGE like PCR-DGGE because different bacterial species may be represented in a single TTGE band (135, 148). Moreover, only dominant species can be observed using universal bacterial primers. Compared to other methods, gel resolution has limitations. For these reasons, the PCR-TTGE band number is generally lower than the number of bacterial species detectable by cultivation-based methods and direct cloning strategies (133, 135, 188). These limitations might account in part for the decreased band number in the present study and may also have influenced the apparent diversity and distance values. Nevertheless, there are several advantages of using molecular tools: they are less time consuming and allowed analysis using frozen samples. Thus the biodiversity could be studied in samples from hospital patients as well as in home samples, as in the present study. In DNA band elution and sequencing experiment, we observed that all sequences from different bands matched with respective species or genus level in genbank except the *Prevotella sp.* The reason might be that the band cut paralleled the marker band of *Prevotella sp.* was not actually in parallel position. So, the wrong bands were sequenced. Although the reason was not clear, we observed higher

concentration of valeric acid in both malnourished children with cholera and without cholera than healthy children. This needs further investigation.

Now a days, molecular tools has improved our knowledge about human gut microbiota more than ever before, still knowledge gap remains in some area and research should be extended. Specially, fluorescent in situ hybridization and TTGE can be combined with direct cloning of 16S rRNA genes from large number faecal samples from children and adults sufferings from diseases of the digestive tract e.g., chronic diarrhea, constipation, irriTable bowel syndrome, dysentery, colon cancer and Crohn's disease. Research can also be done using these metagenomic tools to get an accurate picture about the relation between host genetics and gut microbiota in malnutrition and obesity. Not only in diseases, these techniques can also be applied in health e.g., to monitor the gut microbiota dynamics during intervention of new prebiotic or probiotic substances for children and adults. Real time PCR is a high through put molecular tool and can be applied in gut metagenomics to assess the particular bacterial load during sampling and thus different bacterial abundance can be compared on time periods. At the same time anaerobic isolation techniques for these highly fastidious uncultured bacteria using various media (e.g., with rumen fluid) need to be implemented. Metagenomic tools combined with culture techniques will provide us with many novel taxa as well as will give us a complete picture about the undiscovered area of human gut ecosystem.

The aim of the application of the TTGE method was to evaluate the alterations in bacterial diversity in malnourished children with cholera at different time points, and compare the diversities with non-cholera malnourished and healthy children using a cultivation independent method. Therefore, extensive cloning of the PCR products from chromosomal DNA and cloning of dominant bands during treatment were not suiTable in comparing large number of specimens. Instead, we offered an explanation of the PCR-TTGE profiles related to nutritional status of the study children. We observed a marked difference in number of PCR-TTGE bands between children on standard ORS (glucose ORS) and on other types of ORS additionally containing complex carbohydrates. Although the differences in the bacterial diversity became significant on day 3, there was a trend of higher numbers of bands in the group of children receiving complex carbohydrate-containing ORS beginning on day 1. Mean time to resolution of cholera in our study children was 72 hours, and the magnitude of

the difference in microbiota diversity was observed to evolve from this time point. The results suggested that altered microbial diversity might depend on malnutrition, disease process and type of treatment such as carbohydrate composition of ORS.

The present results may explain the clinical discrepancies observed in previous studies. In previously published clinical trials, amylase-resistant starch was found to be more effective than rice in adults with cholera or other watery diarrhea (157, 158). In well nourished European children with acute non-cholera diarrhea and with mild to moderate dehydration a mixture of non-digestible carbohydrates was ineffective as an adjunct to oral rehydration therapy (98). Those results are at variance with the proven efficacy of rice-ORS in Bangladesh and other countries (3, 7, 84). The discrepancies could be found in the differences in colonic bacteria concentration and pattern; they may also be found in the treatment regimen of the patients, e.g. electrolyte content of ORS, use of antibiotics, schedule and composition of feeding.

So, the present study provides additional scientific basis to reinforce the use of rice-ORS in children with cholera and severe malnutrition. It further suggests that simultaneous measurement of clinical outcome together with bacteria and SCFA concentrations (or in more general terms the metabolic function of the microbiota) may shed light to the too long neglected function of the colonic microbiota in infection and nutrition disorders.

Chapter VII

Conclusion

7.0 Concluding Remarks

1. Bacterial diversity was adversely affected in children with severe malnutrition while acute cholera had little effect on bacterial diversity in these children.

2. Faecal short chain fatty acids concentration was drastically reduced during acute cholera and clinical rehydration over the period was associated with an increase in bacteria and SCFA concentrations.

3. Antibiotic therapy does not affect the species diversity in colon, rather alter the proportion of anaerobic components of faecal microbiota in severely malnourished children with cholera.

4. Complex carbohydrates in oral rehydration solution might favour the proliferation of *Bacteroides sp.* and *Bifidobacterium sp.* in colon of severely malnourished children with cholera.

5. The results of this study might help in further understanding of the better effect of the Rice-ORS in the treatment of cholera.

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9.0 Appendix

Composition of different buffers and solutions:

1. Acrylamide solution for TTGE gel:

Urea ..... 25.2 g

Water ..... 28 mL

50X TAE ..... 1.5 mL

Acrylamide-bis acrylamide (37.5: 1, 40%) 12 mL

The reagents were mixed using magnetic agitation under a light heating and volume was adjusted to 60 mL. Degas was done for 10 minutes and stored at 4°C.

2. Loading buffer:

0.005 g of Bromophenol blue (crystal)

3 ml of water

7 ml of glycerol

3. Electrophoresis buffer for Agarose gel:

Tris base .....10.8 g

Boric acid .....5.5 g

EDTA ..... 0.93 g

Deionized water ..... 1 L

4. Mobile phase for HPLC:

10.8% acetonitrile in 0.007 N H_2SO_4

H_2SO_4 ..... 0.19 ml

Acetonitrile, CH_3CN ..... 108 ml

Deionized water ..... 891.81 ml

5. 0.2 M Acetic acid (standard):

Acetic acid ..... 1.2 µl (from 1g/ml stock)

0.007 N H_2SO_4 ..... 998.8 µl

6. 0.2 M Propionic acid (standard):

Propionic acid ..... 1.48 µl (from 1g/ml stock)

0.007 N H_2SO_4 .....998.52 µl

7. 0.2 M Butyric acid (standard):

Butyric acid ..... 1.76 µl (from 1g/ml stock)

0.007 N H_2SO_4 ..... 998.23 µl

8. 0.2 M Valeric acid (standard):

Valeric acid ..... 2.04 µl (from 1g/ml stock)

0.007 N H_2SO_4 ..... 997.96 µl

9. Cleaning solvent of the column:

5% CH_3CN in 0.005 M H_2SO_4

H_2SO_4	0.083 ml
CH_3CN	15.0 ml
Deionized water	284.92 ml
	300.0 ml

10. Regeneration solvent of the column:

0.025 M H_2SO_4

H_2SO_4	0.416 ml
H_2O	299.584 ml
	300.0 ml

11. Buffer PBS (10X) for FISH

For 100 ml

NaCl	(1M=58)	7.54 g	1.3M
NaH_2PO_4 $2H_2O$	(1M=156)	0.468 g	30 mM
Na_2HPO_4 $12H_2O$	(1M=358)	2.5 g	70 mM

Add 90 ml of H_2O

Adjust the pH at 7.2 with NaOH 1N

Adjust the volume to 100 ml

Filtrate the PBS with 0.22 µM filter in 2 Falcon tubes of 50 ml

The PBS 1X is prepared by dilution in 1/10 of PBS 10X

12. EDTA, 0.5M (pH 8.0) for FISH

Add 18.6 g of di-sodium EDTA $2H_2O$ to 80 ml of H_2O

Adjust the pH with 2 g of NaOH. EDTA will be completely solved when pH reach 8.0

Filtrate with 0.22 µM filter in 2 Falcon tubes of 50 ml

13. Tris HCl, 1M (pH 8.0) for FISH

Add 12.11 g of Trisma base in 90 ml of H_2O

Adjust the pH at 8.0 with HCl 1N

Adjust the volume at 100 ml with H_2O

Filtrate with 0.22 µM filter in 2 Falcon tubes 50 ml

14. NaCl, 5M

Add 29.22g of NaCl in 90 ml of H_2O

Adjust the volume at 100 ml

Filtrate with 0.22 µM filter in 2 Falcon tubes 50 ml

15. Tris-EDTA for FISH

Tris HCl, 1M pH 8	1 ml (100mM)
EDTA, 0.5M pH 8	1 ml (50 mM)
H_2O	8 ml

16. Tris-EDTA-FISH containing Lysozyme

In 10 ml of TE- FISH, add 10 mg of Lysozyme (stored at 4°C).

17. Hybridisation buffer

For 20 ml (volume for 48 tubes)

NaCl	5M	3.6 ml	(0.9 M)
Tris HCl	1M pH 8	400 µl	(20 mM)
SDS	20 %	10 µl	(0.01 %)
H_2O		9.99 ml	

Formamide	(30%)	6 ml

(Formamide was added in a chemical air flow chamber)

18. Washing buffer

For 10 ml

NaCl	5M	128 µl	(0.064M)
Tris HCl	1M pH 8	200 µl	(20 mM)
EDTA	0.5M pH 8	100 µl	(5 mM)
SDS	20 %	5 µl (0.01 %)	
H_2O		9.567 ml	

19. 4% Paraformaldehyde solution

Preparation of 100 ml

PBS 1X ---------- 100 ml
PFA ---------- 4 g
NaOH , 1N ---------- 300 µl
HCl , 1N ---------- Few drops

Heat the sterile PBS ,1X at 60° C in a conical flask. Add PFA in 90 ml of hot PBS , 1X. Add 300 µl of NaOH (to dissolve the PFA at > pH 10.0). Heat at 60° C until the PFA is completely dissolved and agitate (swirl) the flask. (attention: the vapour of PFA is toxic so cover the flask mouth by aluminium foil). Wait for cool down and then adjust the pH to 8.0 by a few drops of HCl, 1N . Add rest of the PBS and keep at 4° C. Don't autoclave or Millipore. Not light sensitive.