EFFECT OF DIET ON PRODUCTION OF BUTYRATE BY THE HUMAN BREAST

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfilment of the Requirements for

the Degree of Master of Science in the

Division of Nutrition and Dietetics

of the College of Pharmacy

University of Saskatchewan

Saskatoon, Saskatchewan

by

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Spring, 1994

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ABSTRACT

The short chain fatty acid, butyrate, is known to be produced in the human large intestine as a result of fibre fermentation, and also in the breast as one of the fatty acids produced de novo during lactation. Recent research indicates that synthesis of fatty acids in the breast occurs only if the maternal diet is low in fat; with typical North American fat intakes, there is adequate fat supply from the mother to maintain milk fat content. In vitro, butyrate has been shown to be inhibitory against cell proliferation in many cancer cell lines, including breast and colon, and is therefore proposed as an anti-cancer agent. Its natural production may therefore represent protection against cancer in these organs. Butyrate is present in cow's milk but there has been little investigation of human milk. A study was therefore undertaken to identify and quantify butyrate in human breast milk, and to study the effect of diet on its production in the human breast.

Twenty-five lactating women kept a record of their diet for a seven day period and expressed 20-50 mL breast milk on the last three consecutive days of the 7 day period. The milk was expressed between 1-2 p.m., using an Egnell breast pump. Samples were immediately frozen and maintained at -70^{0}C until analyzed. One mL of thawed milk was deproteinised using 10% sulphosalicylic acid, followed by vacuum transfer over liquid nitrogen into 250 µL 0.5M NaOH for approximately one hour. The distillate was

freeze-dried overnight. Samples were acidified using 100 µL orthophosphoric acid and immediately injected onto a 30 metre, 0.25mm i.d. acid modified polyethylene glycol capillary column on a Perkin Elmer Sigma 2000 gas chromatograph, with Helium as the carrier gas. 2-Methylvalerate was used as the internal standard. Diet records were analyzed using the NUTS computer program. The mean (± S.E.M) intake of energy was 2238 ± 114 kcals/day. The mean(± S.E.M) fat intake was 32.1 ± 0.9 percent of energy. The mean(± S.E.M) intake of NSP(dietary fibre) was 14.6 ± 4.9 g/day. Butyrate was found in all milk samples tested, quantified for the first time with mean (± S.E.M) values being 14.4 ± 2.1 µmol/L. The mean(± S.E.M) fat content of the breast milk was 4.1 ± 0.3 gram percent.

This study did not show a correlation between dietary fat intake and butyrate concentrations in breast milk (r=0.02, p=0.92) or between the fibre intake and butyrate concentration (r=-0.04, p=0.85) in breast milk. There may be several reasons for this, such as a natural biological variation in butyrate levels, determined by factors as yet unknown, including genetic factors. Also the range of fat intakes may have been too narrow to detect any real differences in butyrate concentrations. All these factors must be investigated before it can be concluded that diet does not play a role in butyrate production by the human breast.

ACKNOWLEDGEMENTS

The author wishes to express gratitude to Dr. Alison Stephen for her guidance,support and continuing encouragement. The author also wishes to thank Drs. Paterson, Hawes and Jones for their support as advisory committee members.

The author wishes to thank the Department of Animal Science for their permission to work there.

A very special thanks to all "moms" who participated in this project and made this study possible.

The author is thankful to the University of Saskatchewan for providing financial assistance in form of a Rhea Ritchie Scholarship.

A special mention for the administrative staff from the Division of Nutrition and Dietetics.

A very special thanks to Luiza, Lakshmi, Paul, Nik, Kathy, MaryRose, Patty, Margaret, Barb and not to mention Tim for making life as a graduate student bearable, infact enjoyable.

Last but not the least, the author wishes to express heartfelt gratitude to Atul, for bearing with her and providing continuous support, especially during writing.

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LIST OF ABBREVIATIONS

EGF..........Epidermal Growth Factor

ER...........Oestrogen Receptor

PUFA.........Polyunsaturated Fatty Acid

MCFA.........Medium Chain Fatty Acid

LCFA.........Long Chain Fatty Acid

SCFA.........Short Chain Fatty Acid

GLC..........Gas Liquid Chromatography

DHA..........Docosahexanoic Acid

IDDM.........Insulin Dependent Diabetes Mellitus

SIDS.........Sudden Infant Death Syndrome

LHRH.........Luteinizing Hormone Releasing Hormone

BMI..........Body Mass Index

NSP..........Non Starch Polysaccharides

C4:0.........Butyric Acid

C6:0.........Caproic Acid

C8:0.........Caprylic Acid

C10:0........Capric Acid

C12:0........Lauric Acid

C14:0........Myristic Acid

C16:0........Palmitic Acid

C18:0........Stearic Acid

CHAPTER 1

INTRODUCTION

1.1 Importance of Breast-feeding

Breast-feeding is the completion of the reproductive physiological cycle in women. It is not just that, but an art which has been passed on from generation to generation. With the advent of urbanization and the formation of nuclear families, this art has not been disseminated as it should have been. Although infants have been breast-fed for centuries, various alternatives such as employment of a wet nurse, feeding with a cup, bottle-feeding with cow's milk, and artificial formulae have been tried. Breast-feeding stands the test of time against all other infant feeding methods, and is still singularly the most common method of feeding infants [Cone, 1981].

Breast milk is the most suitable feed for the baby, as it contains humoral host resistance factors, and has anti-infective and anti-allergic properties [Jelliffe et al.,1977]. These properties of breast milk can be life-savers, especially in poor sanitary conditions. Breast milk is better suited for the infant's growth needs than cow's milk [Jelliffe et al.,1977, Mata, 1978]. Breast-feeding results in psychological bonding between mother and child, causes the return of the uterus to the non-gravid state more quickly than if the mother were to bottle-feed, may offer contraception and is a convenient economical method of feeding the infant [Baum, 1981, Brown, 1982, Kennedy et al.,1989]. Breast-feeding thus can have benefits not only for the baby but also the mother.

This thesis proposes another benefit for the mother, namely a mechanism whereby breast-feeding may offer protection against breast cancer.

1.2 Risk factors for Breast Cancer

In spite of the recent rapid increase in lung cancer mortality, breast cancer remains the most common cause of cancer death in Canadian women [Canadian Cancer Society, 1990]. Of the many risk factors for breast cancer, several are related to the reproductive history. These include parity, age at first pregnancy, age at menarche, lactation and duration of lactation [Kampert et al.,1988, Apter et al.,1989, Franceschi, 1990, Yaum et al.,1988, Helmrich et al.,1980]. In a study done by Kampert et al.[1988] in premenopausal women, late menarche and early first term pregnancy were protective, multiparous women were protected and lean women had more protection than obese women. In a study which extended investigation to the third decade of life, Apter et al.[1989] showed that in a cohort of girls followed for 13 years, early menarche was a risk factor for breast cancer. This was suggested to be due to the fact that women who had early menarche had higher serum oestradiol concentrations than those with later menarche. Hormonal influences, particularly oestrogen, may therefore mediate the effect of reproductive factors on breast cancer risk [Elwood et al.,1980, Stanford et al.,1986].

A current area of research is the production and control of oestrogen receptors (ER) in the breast [Maehle et al.,1989, Hawkins et al.,1988, Stanford et al.,1986]. Breast cancer can be broadly classified into ER positive (+ve) and ER negative (-ve) on the basis of the presence or absence of oestrogen receptors. Factors influencing ER levels are being investigated in vitro, as are relationships between risk factors and ER status [Cooper et al.,1989, Stanford et al.,1987]. An inverse relationship has been found between epidermal growth factor (EGF) and ER in breast tumours [Sainsbury et al.,1985].

The mechanism by which environmental factors may elicit their effects to produce breast cancer remains elusive. Research at present is focused on certain growth factors which appear more frequently or at high levels in breast cancer patients than controls. Various factors being studied are EGF, transforming growth factors α, β (TGFα)& (TGFβ), and Milk Globule Derived Growth Factor (MGDGF1) [Stoschek et al.,1986, Sainsbury et al.,1987, Roberts et al.,1983] which were initially thought to be present only in transforming cancer tissue. Recent research done by Bates et al.[1990] and Walker-Jones et al.[1989] by stimulation of epithelial membrane antigen in human mammary epithelium has shown that these can be isolated from normal healthy tissue too. In fact studies done by Zwiebel et al.[1986]

and Petrides et al.[1985] have identified these factors in human breast milk. Growth factors therefore appear to have important regulatory functions in normal growth of the breast in pregnancy and lactation as well as affecting abnormal growth during mammary tumour formation and progression [Bates et al.,1990].

In regard to diet, fat has been the nutrient most often related to the risk of breast cancer, but there are other factors which may play a significant role [Van'tVeer et al.,1990a, La Vecchia, 1989, Lubin et al.,1981, Goodwin et al.,1987]. These include dietary fibre and β-carotene [Rose, 1990, Van'tVeer et al.,1990b].

This thesis examines a possible mechanism whereby the dietary fat intake of the mother may influence the risk for breast cancer.

1.3 Butyrate and Breast Cancer

Butyric acid is a four-carbon, short-chain fatty acid which is produced by anaerobic fermentation of carbohydrate substrates like dietary fibre and starch in the large intestine [Cummings, 1981, Englyst et al.,1987]. Recent interest in butyrate derives from its in vitro "anti-cancer" characteristics. Butyrate has been shown to have an inhibitory effect on cell proliferation in colonic cancer cell lines, leukaemic lymphocytes, chick fibroblasts and breast cancer cell lines [St.Hilaire et al.,1980, Stolc, 1982, Hagopian et al.,1977, Graham et al.,1988]. Butyrate is thought to act at the molecular and cellular levels by an action on histone hyperacetylation, although other mechanisms have also been suggested [Kruh, 1982]. Butyrate inhibits induction of hormones and other proteins and reverses transformed characteristics to normal morphological and biochemical patterns [Kruh, 1982, McKnight et al.,1980, Koshihara et al.,1981, Boffa et al.,1981].

In the human breast, the alveolar cells of the mammary epithelium synthesize fatty acids for milk synthesis during lactation. Numerous studies have shown that the fatty acid composition of human milk reflects the maternal diet. A study done by Finley et al.[1985] in vegetarian lactating mothers indicated that a high intake of polyunsaturated fatty acids (PUFA) led to production of milk high in PUFA. Studies by Insull et al.[1959] demonstrated that a high carbohydrate, low fat diet caused increased production of medium chain fatty acids (MCFA). This has also been verified in studies done in South Africa by Westhuyzen et al.[1988].

A large proportion of the literature on human milk fatty acid composition deals with long chain fatty acids (LCFA). Very few studies have reported the content of fatty acids less than C10:0. This is mainly because SCFA and MCFA are lost during methyl ester formation prior to gas liquid chromatography (GLC) analysis or elute too quickly to be detected . Butyrate is known to be present in cow's milk but very few studies mention its presence in human milk [Bracco et al.,1972]. In 1985, Thompson & Smith detected butyrate in breast milk. It was found to be present at 0.2 mol % of milk fat, with milk fat being 4.5 g/100 mL of breast milk. They suggested that the low level of butyrate and other short chain and medium chain fatty acids in human milk could be attributed to the high fat content of maternal diets in North America which cause a repression of de novo synthesis of milk fat by the breast.

Sources for production of fatty acids include glucose, lactose, ketone bodies, SCFA and other lipids [Bartley et al., 1976]. In ruminants as well as monogastrics, acetate is produced as a result of extensive fibre fermentation in the intestine, and is known to be a major precursor of fatty acid synthesis [Apter et al.,1989]. Thus a low fat, high fibre diet may facilitate fatty acid synthesis in the human breast [Thompson et al.,1985]. The capability of the human breast to produce SCFA & MCFA is beneficial to the infant as these fatty acids can be easily digested and metabolised by the infant as compared to LCFA [Bach et al.,1982].

This thesis suggests that the production of butyrate in breast milk, as well as being beneficial for the infant, may also be beneficial to the mother.

1.4 Thesis Objective

To study the production of the short-chain fatty acid butyrate by the human breast, and the effect of diet on its level in breast milk.

1.5 Thesis Hypothesis

The hypothesis to be tested in this study was that butyrate is produced by the human breast during lactation and that its content may be affected by the level of fat in the maternal diet.

1.6 Thesis Overview

This thesis is divided into six chapters, with this chapter acting as an introduction to the thesis topic. An overview of the rest of the thesis is given below:

Chapter 2 deals with the importance of breast-feeding to infant and mother, synthesis and significance of SCFA & MCFA and specifically the mode of action of butyrate. This chapter presents the rationale behind the thesis supported by a literature review.

Chapter 3 discusses the methodology used for data collection.

Chapter 4 presents the results of the study undertaken.

Chapter 5 includes a discussion of the outcome of the study.

Chapter 6 concludes the thesis. It also suggests directions for future work in this area.

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of Breast-feeding

2.1.1 History of Breast-feeding

The presence of mammary glands is a distinguishing feature of class mammalia to which humans belong. It is therefore not surprising that breast-feeding is a crucial component of the many physiological processes in the human life-cycle. Infant-feeding has been achieved through a number of different ways: employment of a wet nurse, use of spouted pots of pewter, silver, other available metals and glass bottles.

The job of a wet nurse was filled with superstition and governed with laws and codes of ethics. Before the 18th century, Phaire, like his contemporaries believed that both good and bad characteristics of the wet nurse could be transmitted through her milk to the infant, e.g. if she were a red head, a treacherous and ill-temper would be transmitted [Cone, 1981]. Around the mid-1700s, mothers used spouted pots to feed "pap", a mixture of bread and some liquid, either milk or water. The result was such that if the mother could not or refused to breast-feed her child, it meant sure death for the child. Around the 19th century, introduction of the glass bottle along with milk pasteurization and water chlorination made artificial feeding safer. Cow's milk was thought to be the proper, hygienic and civilised food for infants. By the 20th century, consumption of cow's milk was encouraged by the government by subsidizing the sale of National Dried Milk [Pierse et al.,1988]. By the mid-sixties only one out of six mothers was breast-feeding her infant. Even medical practitioners took a passive role, indirectly supporting bottle feeding. The 1970's saw a return of breast-feeding, particularly among professionals. It came in vogue in the upper strata and slowly trickled into the lower strata which was then bottle feeding.

Efforts have been made to duplicate human milk by modification of cow's milk, but not with complete success because human milk contains nutritive and non-nutritive components, enzymes, hormones and immunological factors all of which may not be present in cow's milk [Jelliffe et al.,1977].

2.1.2 Advantages to the Infant

1] <u>COLOSTRUM</u>:

Breast milk in the first 3-4 days is called colostrum. It is higher in protein content as well as total ash as compared to mature human milk. During the first two weeks of lactation, the concentrations of proteins and fat soluble vitamins decrease, whereas lactose, fat, water soluble vitamins and energy increase [Blanc,1981]. Colostrum provides the infant with the required nutrition and is a good source of immunoglobulins which protect the infant from enteric infections [Goldman et al.,1986]. Hanson et al.[1981] have shown that breast-feeding results in lower frequency of infection in the infant not only in developing countries but even in Canada and the US. The large number of infections, especially diarrhoea, that could follow bottle-feeding may be a major factor in impairing growth and development with accompanying undernutrition especially in the developing countries.

2] NUTRITION:

Fat:

Fat content in human milk ranges from 3-5 g/100mL [Pierse et al.,1988]. The fat concentration in human milk seems to vary within feeds, diurnally, in response to the diet and with stage of lactation [Hall,1979]. Bitman et al.[1983] have reported an increase in fat content as the day progresses from morning to evening. The concentration of total lipid can also be as much as doubled from the beginning to the end of the feed [Neville et al.,1984]. Fat exists in human milk mainly as triglycerides. The fatty acid composition of human milk and the position of fatty acids on the glycerol molecule facilitate fat absorption by infants as compared to cow's milk. The digestibility of the milk fat is increased by the fact that the palmitate residue is mainly in the second position of the glycerol molecule. Fat absorption may also be helped by significant lipolytic activity in human milk [Hernell,1975]. The major components are palmitic, stearic, oleic and linoleic acid. However Jensen et al.[1978] have reported finding traces of butyric acid (C4:0) in human milk fat. Milk obtained from vegetarian women has been shown to have a higher concentration of PUFA [Finley et al.,1985] as compared to that from non-vegetarian mothers, though the amount of total fat is comparable. PUFA levels in human milk constitute 14% of the total fat on average; however infant formulas contain two-three times more linoleic acid. Feeding such high PUFA levels in preterm infants may cause deficiency of vitamin E as a result of increased lipid peroxidation [Williams et al.,1975]. Medium-chain triglycerides (MCT) present in breast milk are ideal for fat absorption. MCT, C8:0, C10:0, C12:0, C14:0 are easily hydrolysed by lipases, absorbed and can enter the mitochondrion without carnitine, and are a good source of ketone bodies which are a major source of energy for brain development. It has been shown that MCT can increase calcium absorption and induce weight gain in pre-term infants, and that feeding infants milk lacking MCT can cause poor fat absorption, leading to difficulty in meeting energy requirements and nitrogen retention [Roy et al., 1975].

Docosahexanoic acid (DHA), an essential omega-3 fatty acid found in breast milk as well as in the infant's brain and retinal tissue is absent in formulae [Simopoulos,1991]. Some formulae contain α-linolenic acid which infants can partially convert to DHA, however premature infants have a limited capacity to do so. In a study done by Carlson [1989], preterm infants not fed human milk had plasma DHA levels similar to monkeys fed safflower oil, leading to deficits in visual acuity in monkeys.

Cholesterol:

The cholesterol content of human milk is 200-330 mg/100g fat [Pierse et al.,1988]. Cholesterol helps in the formation of nerve tissue in the central nervous system and is required for synthesis of steroid hormones. Infant formulas contain vegetable oil only and therefore lack cholesterol. Studies done by Reiser et al.[1972] and Hahn et al.[1973] in animals indicate that ingestion of cholesterol during infancy may help in inducing enzymes for cholesterol metabolism, which may help in later life to keep serum cholesterol levels low. A study from Boston has shown that 30-year-old adults who had been exclusively breast-fed for at least two months had lower serum cholesterol levels than those who had been breast-fed for a shorter duration than two months [Glueck et al.,1972]. However this has not yet been confirmed in some animal studies and in retrospective studies done in infants by Freidman et al.[1975], a high cholesterol feeding did not protect the infant against a high cholesterol level in later life. Infant feeding studies done by Hodgson et al.[1976] concluded that milk with lower cholesterol and higher PUFA early in life led to lower serum cholesterol levels later in life.

Protein:

The protein content in human milk is nearly one-third of that in cow's milk. This is beneficial to the infant; because of the lower amount of protein consumed, the infant's kidneys can excrete urea, the end product of protein metabolism easily. The lower protein content in human milk is very well suited for the metabolic activity of the newborn infant, particularly a preterm infant, because the liver may be inefficient in converting methionine to cysteine and metabolising tyrosine [Raiha,1974]. The low protein concentration contributes to a low renal solute load [Rolfes, 1990]. The whey: casein ratio is 20:80 and 65: 35 in cow's and human milk, respectively. The ratio in human milk is better suited for the infant as the higher whey content constituted by α-lactalbumin, contains the "immunologically significant" proteins. α-lactalbumin is also richer in sulphur containing amino acids like methionine. The whey component in cow's milk is made up of β-lactoglobulin which is viewed as an allergenic protein [Nutrition Committee of the Canadian Paediatric Society and the Committee on Nutrition of the American Academy of Pediatrics,1978]. The high casein content in cow's milk forms curds which are difficult to digest and may cause discomfort to the infants. Taurine, an amino acid that has recently gained interest, is abundant in human milk and virtually absent in cow's milk [Lawrence,1985]. Breast milk also contains nucleotides which provide a source of non-protein nitrogen which may play an important role in the growth of the infant. In human milk, 25% nitrogen is supplied by non-protein nitrogen whereas in cow's milk only 6% is supplied as non-protein nitrogen. The nutritional significance of non protein nitrogen has yet to be elucidated [Nutrition Committee of the Canadian Paediatric Society and the Committee on Nutrition of the American Academy of Pediatrics, 1978].

Minerals:

The mineral content of human milk is well suited for infant needs. Calcium, sodium and potassium content in human milk is about one third that in cow's milk. Though the calcium content in cow's milk is higher than human milk, it is less bioavailable and infants fed on cow's milk are known to suffer from neonatal hypocalcemia [Roberts et al.,1973]. Formula fed infants have a 30/10,000 risk of hypocalcemia as compared to a risk of 1/10,000 in breastfed infants [Specker et al.,1991]. The renal solute load in human milk is 7.5 Osm/dL as compared to 23 Osm/dL and 11 Osm/dL in cow's milk and artificial formulae, respectively. These higher osmolarities may lead to vomiting, fever, diarrhoea and dehydration.

The iron content of human milk is 0.3-0.5 mg/L, which is low compared to milk of other animals. Formula-fed infants receive twenty times more iron than that found in human breast milk, which causes concern about its interference with absorption of trace minerals and the infant's resistance to infection. A higher concentration of iron would cause loss of bacteriostatic effects in proteins like lactoferrin and transferrin due to saturation with iron. However consumption of low-iron formulas by infants can cause iron deficiency leading to uncorrectable cognitive delays [Lozoff et al.,1991]. McMillan et al.[1976] report that iron in human milk is sufficient to meet the iron requirement of exclusively breast-fed infants until they triple their birth weight. Studies done by Wilson et al.[1974] show that infants are prone to iron deficiency because of being fed cow's milk early in life because cow's milk is a poor source of iron and partly because cow's milk which has not been adequately heat-treated causes significant gastrointestinal blood loss in infants. Term infants that are exclusively breastfed do not need iron supplementation until about 6 months of age after which iron fortified infant cereals should be introduced [Nutrition Committee, Canadian Paediatric Society,1991].

Zinc in human milk is bound to whey protein and thus has a higher bioavailability [Eckhart,1985]. In cow's milk zinc is present in nearly twice the amount however bound to the casein fraction.

Vitamins:

Deficiency of water soluble vitamins is rare in breastfed infants. Thiamine deficiency is seen only when the mother has an unfortified thiamine depleted diet. Vitamin C deficiency is rarely seen. Vitamin A concentration in breast milk is maintained at the cost of mother's stores for as long as they last. Studies show that vitamin A deficiency sets in if breast-feeding is stopped in early childhood and proper supplementation is not given to the infant [West et al.,1986]. Vitamin D has to be supplemented either by diet or sunlight but excessive amounts can be toxic. In a recent study in Massachusetts on vitamin D concentrations in formula fed infants, 7 of 10 samples contained more than 200 percent of the stated amount of vitamin D [Holick et al.,1992].

Formulae that approximate the composition of human milk are widely used as the sole source of nutrients as replacement for breast milk. Such formulas contain cow's milk, soymeal proteins or protein hydrolysates together with forms of fat, carbohydrate, vitamins and minerals that are bioavailable to infants. Problems of nutrient deficiency are now almost unknown with formulae so closely resembling human milk, but adverse reactions of individuals to specific formula ingredients are still seen [Anderson et al.,1982].

3] <u>IMMUNITY</u>:

Anti-infectious properties:

Various infectious diseases are reported to be less frequent in breastfed infants [Cunningham,1977, Mellander et al.,1959, Winberg et al.,1971]. A study done by Adebonjo[1972] in a small affluent community showed no difference between breastfed and bottlefed infants in terms of resistance to infection. Another study suggests that the protective effect of breast-feeding against diseases is seen only when breast-feeding is an ongoing process[Larsen et al.,1978]. It is known that the infant acquires certain immunity from the mother, transplacentally, and receives protection against enteric infections through breast milk. An observation by Goldblum et al.[1975] suggests the presence of an entero-mammary system by which enteric antigens stimulate gastric mucosal cells in the mother to migrate to the breast tissue and secrete antibodies.

Breast-feeding has been shown to prevent gastroenteritis in developing countries and, although less common, in developed countries. Duffy et al.[1986] have shown that breast-fed infants even in industrialized countries are less susceptible to infection, whereas the formula-fed infants have a 3-4 fold risk of diarrhoeal illness. Breast-feeding offers protection against respiratory diseases [Wright et al.,1989].

4] <u>HYGIENE</u>:

Breast-feeding eliminates the dangers of contamination during unsanitary bottle feedings especially in the developing countries. The infant formulae are constituted to meet the nutritional requirements of infants, making them as close to human milk as possible. To reconstitute these formulae, water or milk may have to be added and if this water is not safe for drinking, there is a chance of contamination, thereby exposing