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(54) Title: BACTERIAL OR YEAST EXTRACTS WHICH STIMULATE THE PRODUCTION OF DEFENSINS AND METHODS OF USE THEREOF (57) Abstract The subject invention relates to extracts derived from, for example, a spent medium, a portion of a bacterial cell, a lysed yeast cell, or a portion of a lysed yeast cell which may be used to stimulate the production of defensins in eukaryotic cells such as, for example, mammalian cells. Furthermore, the present invention includes compositions containing these extracts as well as methods of using these extracts, for example, in the prevention and treatment of various disease states, in rehydration, and in the stimulation of the immune system.		

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BACTERIAL OR YEAST EXTRACTS WHICH STIMULATE THE PRODUCTION OF
DEFENSINS AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

5 Technical Field

The subject invention relates to extracts derived from, for example, a cell-free culture medium that has been used for growing yeast or bacteria (i.e., "spent medium"), a portion of a bacterial cell, a lysed yeast cell, a portion of a lysed yeast cell, or a mixture thereof, which may be used to stimulate the production of defensins in eukaryotic cells such as, for example, mammalian cells. Furthermore, the present invention includes compositions containing these extracts as well as methods of using these extracts. For example, the extracts may be used in the prevention and treatment of various disease states.

Background Information

Defensins are antimicrobial peptides that contain six cysteines (Ganz et al., "Defensins" in Pharmac. Ther., assoc. ed., D. Grunberger, Elsevier Science, Great Britain, Vol. 66, pages 191-205, 1995). More specifically, defensins are peptides that are approximately 29-45 amino acids in length, contain three pairs of disulfide-linked cysteines, and are cationic because of their high arginine content (Martin et al., Journal of Leukocyte Biology 58:128-136 (1995)). There are three types of defensins: classical, beta and insect (Ganz et al., supra). (Beta-defensins will be discussed, in detail, below.) The three groups differ with respect to the spacing and connectivity of their six cysteine residues (Ganz et al., supra).

Classical defensins are mammalian peptides which are stored in high concentrations in granules of phagocytic and Paneth cells (Ganz et al., supra). They are active against gram positive and gram negative bacteria, spirochetes, mycobacteria, fungi, and certain enveloped viruses (Martin et al., supra; Patterson-Delafield et al., Infect. Immun. 30:180-192 (1980); Lehrer et al., Infect. Immun. 42:10-14 (1983); Lehrer et al., J. Virol. 54:467-72 (1985); Selsted et al., Infect. Immun. 55:2281-86 (1987); Selsted et al., Infect. Immun. 45:150-54 (1984); Selsted et al., Infect. Immun. 49:202-206 (1985); Ganz, T., Infect. Immun. 55:568-71 (1987); Segal et al., J. Infect. Dis. 151:890-94 (1985); Daher et al., J. Virol. 60:1068-1074 (1986); Levitz et al., J. Infect. Dis. 154:483-489 (1986); Greenwald et al., Infect. Immun. 55:1365-68 (1987); Shafer et al., Infect. Immun. 56:51-53 (1988); Eisenhauer et al., Infect. Immun. 57:2021-27 (1989); Yamashita et al., Infect. Immun. 57:2405-2409 (1989); Cullor et al., Arch. Ophthalmol. 108:861-64 (1990); Miyasaki et al., Infect. Immun. 3934-40 (1990); Borenstein et al., Infect. Immun. 59:1359-67 (1991); Kohashi et al., Microbiol. Immun. 36:369-80 (1992); Ogata et al., Infect. Immun. 60: 4720-25 (1992); Wu et al., Hua. Hsi. I. Ko. Ta. Hsueh. Hsueh. Pao. 23:126-29 (1992); Nakashima et al., AIDS 7:1129 (1993)).

Additionally, defensins may be cytotoxic to mammalian cells in vitro and may also contribute to neutrophil-mediated antibody-dependent lysis of tumor cells (Lichtenstein et al., Blood 68:1407-10 (1986); Am. Rev. Respir. Dis. 141:179-85 (1990); Barker et al., Cancer Res. 53:362-67 (1993)).

Furthermore, defensins may also modulate inflammation and

repair (Ganz et al., "Defensins" in Pharmac. Ther., assoc. ed., D. Grunberger, Elsevier Science, Great Britain, Vol. 66, pages 191-205, 1995).

As noted above, three types of defensins exist. One type is the beta-defensins. More specifically, beta-defensins are a specific structural class of defensins, and like most defensins, they are broad spectrum antimicrobial peptides. They were first discovered in the epithelia of cows (Diamond et al., Proc. Natl. Acad. Sci. USA 88: (1991)), but have subsequently also been found in the epithelia of humans (Zhao et al., FEBS Letters 396:319-322 (1996); Harder et al., Nature 387:861 (1997); Goldman et al., Cell 88:553-560 (1997)). They are present, most likely, in most mammalian epithelia. Three beta-defensins have been described in bovine epithelia including tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide (LAP) (Schonwetter et al., Science 267:1645-1648 (1995)), and enteric beta-defensin (EBD) (Tarver et al., J. Infect. Immun. 66:1045-1056 (1998)). Beta-defensins are also present in neutrophils in cattle (Selsted et al., J. Biol. Chem. 263:9573-9575 (1993)), but have not been reported in human neutrophils. The beta-defensins in cattle are coded for by a related gene family. These genes exhibit very similar nucleic acid sequences and therefore experimental nucleic acid reagents, based on the sequence of one family member, will in many cases also detect or hybridize to other members of the bovine beta-defensin family. Two human beta-defensins have been reported, human beta-defensin-1 (HBD-1) (Bensch et al., FEBS Letters 368:331-335 (1995)) and human beta-defensin-2 (HBD-2) (Harder et al., Nature 387:861 (1997)). Expression of many beta-defensins is highly

inducible in epithelia, and this is also true of other classes of antimicrobial peptides, including those found in humans (Frohm et al., J. Biol. Chem. 272:15258-15263 (1997)).

Due to the many significant host defense properties of defensins, any means which stimulates or induces the production of these peptides is desirable and certainly much needed. The present invention provides such a means as well as uses and compositions relating to defensins.

SUMMARY OF THE INVENTION

The present invention includes a composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of such a yeast cell. The composition may contain mixtures of any one of these entities or members, for example, different portions of different bacterial cells or yeast cells, or mixtures of the different entities or members. An "extract", for purposes of the present invention, is defined as a solution comprising, for example, a portion of a bacterial cell, the medium in which a bacterial cell is grown, a lysed yeast cell or a portion thereof, or mixtures thereof. The extract may contain, for example, cell wall components, cell surface components, cell membrane components, secreted products, the cell nucleus, and the endoplasmic reticulum. Other components of bacterial or yeast cells may also be present in the extract. The bacterial cells may be, for example, of the genus Lactobacillus or Bifidobacterium. In particular, the bacterial cells may be Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium infantis, or

Lactobacillus casei cells. The yeast cells may be, for example, Kluyveromyces spp., Saccharomyces spp. or Hanensula spp. cells. In particular, the yeast cells may be Saccharomyces cerevisiae or Saccharomyces boulardii cells.

The composition may be, for example, a pharmaceutical
5 composition or a nutritional composition, and it may be either in the form of a solid or a liquid.

The present invention also includes a method of eliciting the production of defensins by eukaryotic cells. This method
10 comprises exposing the eukaryotic cells to a composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of the yeast cell. The extract is as described herein. The
15 defensin molecules which may be stimulated are present in eukaryotic cells such as mammalian cells and, more particularly, are present in epithelial cells.

The extracts of the present invention have been shown to
20 stimulate defensin molecules of the type found in, for example, a human tissue or source selected from the group consisting of the tongue, buccal mucosa, nasal mucosa, the conjunctiva, the choroid plexus, the trachea, the bronchi, the lung, the fallopian tubes, the uterus, the cervix, the vagina,
25 the testes, the bladder, the urethra, the esophagus, the duodenum, the jejunum, the ileum, the caecum, the ascending colon, the descending colon, the rectum, the brain, the kidney, the heart and the spleen. Additionally, the extracts of the present invention have been shown to stimulate defensin
30 molecules of the type found in, for example, an animal tissue

or source selected from the group consisting of the utter, the rumen, the reticulum, the omasum, the abomasum, and the spiral colon.

Additionally, the present invention encompasses a method of screening for a defensin-inducing material comprising the steps of: 1) adding the sample to be tested for defensin-inducing activity to a cell line, 2) isolating the total RNA from cells of the cell line, 3) quantifying the total RNA, 4) creating total cDNA by reverse transcription of total RNA, 5) amplifying the resulting beta-defensin cDNA, 6) analyzing the amount of beta-defensin cDNA present in comparison to negative control cDNA, a higher amount of beta-defensin cDNA relative to control cDNA indicating a test sample which contains defensin-inducing material.

Also, the present invention includes a method of identifying up-regulators of defensins and, in particular, beta-defensins, comprising the steps of: 1) constructing an expression vector comprising a defensin gene promoter operably linked to a reporter gene, 2) transfecting a host cell with the expression vector, 3) culturing the transfected host cell in the presence of the test sample, and 4) measuring the level of mRNA or reporter gene expression against a control, a higher level of mRNA or reporter gene expression relative to said control indicating the presence of an up-regulator in the test sample.

Furthermore, the present invention includes a method of treating or preventing a disease state in a patient. This method comprises administering a composition comprising the

extract described above, in an amount sufficient to effect treatment or prevention. The disease state may be caused by any viral, bacterial or fungal pathogen including, for example, Candida albicans, Rotavirus, Respiratory Syncytial Virus or E. coli.

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Additionally, the invention includes a vaccine comprising an adjuvant, wherein the adjuvant comprises an extract derived from at least one member selected from the group consisting of a cell-free culture medium used for growing a bacterial cell, a portion of a bacterial cell, a lysed yeast cell, and a portion of the lysed yeast cell. The vaccine may be administered enterally or parenterally (e.g., subcutaneously, intramuscularly, topically, etc.). If parenteral administration is via a mucosal route, oral, rectal, vaginal or pulmonary administration may be used. The vaccine may be administered to humans or to animals.

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Moreover, the invention also encompasses a method of stimulating the immune system of a mammal comprising administering a composition, to the mammal, comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell and a portion of the yeast cell, in an amount sufficient to effect the stimulation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the defensin stimulation assay described in detail in Example I.

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Figure 2 represents the relative defensin expression in

test cells upon exposure to various fraction of spent L. acidophilus bacterial culture medium.

Figure 3 illustrates the induction of defensin expression by bacterial filtrates.

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Figure 4 represents the dose response of defensin induction by a 1 Kd filtrate from Lactobacillus acidophilus.

Figure 5 illustrates the induction of HBD-2 human
10 keratinocyte defensin expression as a result of exposure to a 1 Kd filtrate from Lactobacillus acidophilus.

Figure 6 shows a chromatogram of the flow-through material recovered from a reverse-phase C18 column when the 1
15 Kd filtrate from L. acidophilus was loaded on the column. This material was active in the defensin stimulation assay.

Figure 7 is a chromatogram which evidences that a 1 Kd filtrate contains material which eluted with 5% acetonitrile
20 from a C18 column. The material contained the active ingredient.

Figure 8 is a chromatogram of the material that eluted from the C18 column with 10% acetonitrile wash after the
25 material shown in Figure 7 had been eluted with a 5% acetonitrile wash. The material was inactive in the defensin stimulation assay.

DETAILED DESCRIPTION OF THE INVENTION

30 The subject invention relates to a purified extract

derived from 1) a portion of a bacterial cell, 2) a lysed yeast cell, 3) a portion of the lysed yeast cell, 4) a spent medium (i.e., a cell-free culture medium that has been used for growing bacteria or yeast), or 5) mixtures thereof, as well as to compositions comprising these extracts. The composition may be used for various purposes. More specifically, the composition may be used to stimulate the production of defensins in eukaryotic cells, to prevent or treat various disease states or conditions, or to rehydrate a patient. As an example, a composition containing the purified extract may be used to reduce colonization of, for example, Candida albicans in mammals.

More specifically, the present invention relates to stable extracts derived from Bifidobacterium sp., Lactobacillus sp., Kluyveromyces spp., Saccharomyces spp. and/or Hanensula spp. and, more preferably, Bifidobacterium bifidum, Bifidobacterium infantis, Lactobacillus casei, Saccharomyces cerevisiae, Saccharomyces boulardii and/or Lactobacillus acidophilus, which may be used as components of nutritional or pharmaceutical compositions. Cell wall components, cell surface components, cell membrane components, secreted products and the endoplasmic reticulum, for example, may be the actual components or portions of the bacterial and/or yeast cells utilized in the extract.

In particular, the present invention encompasses a composition comprising an extract derived from at least one member selected from the group consisting of a cell-free medium used for growing a bacterial cell, a portion of a bacterial cell, a lysed yeast cell or a portion of the lysed

yeast cell. Mixtures of one of these entities may be used in the composition (e.g., cell surface components and components of the cell nucleus). Mixtures of the separate entities (e.g., the cell medium and a bacterial cell surface antigen) may also be utilized in the composition.

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The invention also relates to methods of using these extracts. As noted above, such extracts have the ability to stimulate the production of defensins in eukaryotic cells and therefore may be used in the treatment or prevention of various disease states or conditions. Consequently, a method of treating or preventing a disease state in a patient may comprise administering the composition comprising the extract, to the patient, in an amount sufficient to effect the treatment or prevention.

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As noted above, the extracts of the present invention also have the ability to stimulate the production of defensins in eukaryotic cells. Thus, the present invention also includes a method of eliciting the production of defensin by eukaryotic cells. This method comprises exposing the cells to the composition described above in an amount sufficient to elicit the production of the defensins by the cells.

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Additionally, the present invention includes a rehydration composition comprising the extracts of the present invention. Such solutions, in addition to rehydrating the patient and restoring electrolyte balance, for example, will also stimulate the production of defensins in the epithelial cells of the patient. One solution to which the extracts may be added is Pedialyte™. It should be noted that the extract

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may be added to a rehydration solution which will ultimately be produced in either a liquid or solid form.

Further, the present invention also includes a method of rehydrating a patient. This method includes administering the rehydration composition described directly above to a patient in need of rehydration. The composition is administered in an amount sufficient to effect rehydration.

The purified extracts of the present invention are cell free and therefore do not contain live or dead bacteria. They have a molecular weight of less than 1000 daltons and are soluble (i.e., dissolve in aqueous solution), unlike probiotic bacteria. Furthermore, it should be noted that the extracts do not contain the bacterial cell wall component glycosylated muramyl di-peptide (GMDP).

Furthermore, the extracts of the present invention have been shown to stimulate defensin molecules of the type found in epithelial cells derived from the following mammalian tissues or sources: tongue, bucal mucosa, nasal mucosa, conjunctiva, choroid plexus, trachea, bronchi, lung, fallopian tubes, skin, uterus, cervix, vagina, utter, testes, bladder, urethra, esophagus, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, caecum, ascending colon, spiral colon, descending colon, rectum, brain, kidney, heart and spleen. The extracts may also be used to stimulate epithelial cells found in other tissues or sources, for example, the ear, the liver, the pancreas and the ovary. Consequently, the extracts may therefore be utilized to treat or prevent, for example, skin, oral, eye, ear, respiratory, gastrointestinal,

colorectal and urinary diseases or other epithelial cell-related diseases or states in mammals, including humans and animals. Possible routes of administration include but are not limited to, for example, oral and parenteral (e.g., intramuscular, topical, and subcutaneous) administration. The route of administration is chosen based upon the condition one is trying to treat or prevent. For example, if a skin disease or condition is present, one may wish to use perhaps topical administration.

The dosage of extract to be administered may be readily determined by one of ordinary skill in the art and depends upon various factors such as weight of the patient, age of the patient, immune status of the patient, etc. If administered parenterally, the composition containing the extract will typically contain a standard, well-known, nontoxic, pharmaceutically acceptable carrier, adjuvant or vehicle such as, for example, phosphate buffered saline, water, wetting agents, and emulsions such as oil/water or water/oil.

Additionally, the present invention includes vaccines in which the above extract is utilized as an adjuvant. Such vaccines may be administered orally or parenterally to mammals including humans and animals. Such parenteral administration may be via a mucosal route, for example, via an oral, rectal, vaginal or pulmonary route.

Furthermore, the present invention includes a method of stimulating the immune system of a mammal after, for example, surgery, immune ablation by chemotherapy or other treatments, or bacterial or viral infections. Such a method comprises

administering a composition comprising the extract of the invention to the patient (i.e., human or animal) in need of immune system stimulation in an amount sufficient to effect such stimulation. Thus, the composition stimulates the production of defensins in the epithelial cells of the patient.

Also, the present invention includes a method for screening for a defensin-inducing material by use of a stable cell line. This method comprises the steps of: 1) adding the sample to be tested for defensin-inducing extracts to an immortal cell line, for example, Madin-Darby Bovine Kidney (MDBK) cells, 2) isolating the total RNA from the cells, 3) quantifying the total RNA, 4) creating total cDNA by reverse transcribing the total RNA, 5) amplifying the resulting beta-defensin cDNA using, for example, the polymerase chain reaction, and then 6) analyzing the amount of beta-defensin cDNA present in comparison to the negative control cDNA, a higher amount of beta-defensin cDNA than control cDNA indicating a test sample which contains defensin-inducing material. A cell line such as a MDBK cell line may be used indefinitely in the method. In the past, others have used only primary cell lines which have limited viability.

Additionally, the present invention also encompasses a method of identifying up-regulators of beta-defensins or other mammalian antimicrobial peptides. Up-regulators of beta-defensins can be identified by constructing an expression vector containing a beta-defensin gene promoter operably linked to a reporter gene (i.e., a gene producing an easily assayed protein, for example, luciferase) transfecting a host

cell with the expression vector, and culturing the host cell in the presence of test substances. Whether the test substance is an up-regulator is then determined by measuring the level of mRNA or reporter gene expression.

5 The present invention may be illustrated by the use of the following non-limiting examples:

EXAMPLE I

DETERMINATION OF DEFENSIN STIMULATORY ACTIVITY OF VARIOUS TEST 10 SAMPLES

A. Preparation of Test Samples:

The following test samples were utilized:

Proteins:

Bovine lactoferrin	Soy flour
20 Human lactoferrin	Rice flour
Alpha-lactalbumin	Bovine serum albumin
Human kappa-casein	Whey protein concentrate
Bovine kappa-casein	Casein hydrolysate
Bovine glycomacropeptide	Proteose peptone
25 Soy protein hydrolysate	Lactopep G
Soy PP750	Pea protein
Soy PP1610	Pea protein hydrolysate
Soy Ardex-F	Soy collagen
30 Defatted soy flakes	Yeast autolysate

Carbohydrates/fibers:

Transgalactosylated Oligosaccharide (TOS) - supplier 1
 Transgalactosylated Oligosaccharide (TOS) - supplier 2
 35 (Lactose G)
 Fructooligosaccharide (FOS) - supplier 1
 Fructooligosaccharide (FOS) - supplier 2 (FOS-P)
 Xylooligosaccharide (XOS)
 Lacto-N-neotetrose (LNnT)
 40 Lacto-N-fucopentose I (LNF I)
 Lacto-N-fucopentose II (LNF II)

3'-Sialyllactose
6'-Sialyllactose
Maltrin M100
Gum arabic
Carboxymethyl cellulose (CMC; low density)
5 Yeast glucans
Guar gum
Karaya gum
Locust bean gum

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Bacterial strains/media:

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Bifidobacterium bifidum ATCC 15696-grown in complex medium
Bifidobacterium bifidum ATCC 15696-grown in semi-synthetic medium
Bifidobacterium infantis ATCC 15697-grown in complex medium
Bifidobacterium infantis ATCC 15697-grown in semi-synthetic medium

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Bifidobacterium breve ATCC 15700-grown in complex medium
Bifidobacterium breve ATCC 15700-grown in semi-synthetic medium
Bifidobacterium longum ATCC 15708-grown in complex medium
Bifidobacterium longum ATCC 15708-grown in semi-synthetic medium

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Lactobacillus acidophilus ATCC 4356-grown in complex medium
Lactobacillus acidophilus ATCC 4356-grown in semi-synthetic medium

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Lactobacillus casei var. GG ATCC 53103-grown in complex medium
Lactobacillus casei var. GG ATCC 53103-grown in semi-synthetic medium
Lactobacillus reuteri ATCC 23272-grown in complex medium
Lactobacillus reuteri ATCC 23272-grown in semi-synthetic medium

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Bacteroides ovatus ATCC 8483-grown in complex medium
Bacteroides ovatus ATCC 8483-grown in semi-synthetic medium
Bacteroides fragilis ATCC 43858-grown in complex medium
Bacteroides fragilis ATCC 43858-grown in semi-synthetic medium
Escherichia coli O111 ATCC 43887-grown in complex medium (aerobic)

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Escherichia coli O111 ATCC 43887-grown in complex medium (anaerobic)
Campylobacter jejuni ATCC 29428-grown in complex medium
Streptococcus pneumoniae ATCC 6303-grown in complex medium

Complex medium control for *Bifidobacterium* spp. and *Bacteroides* spp.

Complex medium control for *Lactobacillus* spp.

Complex medium control for *Escherichia coli* O111

Complex medium control for *Campylobacter jejuni*

5 Complex medium control for *Streptococcus pneumoniae*

Semi-synthetic medium control for *Bifidobacterium* spp.,
Lactobacillus spp., and *Bacteroides* spp.

10 The above samples were prepared as follows:

Proteins:

The protein samples were dissolved in distilled water and
sterilized by passage through a 0.22 u filter. The protein
15 concentration of the sterile solution was determined by UV
absorbance, and an appropriate amount was dried under vacuum.

Carbohydrates/Fibers:

TOS (supplier 1) and XOS were obtained as syrups. These
20 samples were weighed to determine the density, and the syrups
were then sterilized by autoclaving. An aliquot of the
sterile syrup was sent for testing. Samples of guar gum,
karaya gum, and locust bean gum were weighed and dissolved in
distilled water. These syrups were then sterilized by
25 autoclaving. An aliquot of the sterile syrup was sent for
testing.

The remainder of the carbohydrate and fiber samples were
weighed and dissolved in distilled water. The solutions were
30 sterilized by passage through a 0.22 u filter. An appropriate
amount of the solution was then dried under vacuum.

Bacterial strains/media:

The *Bifidobacterium* spp. and *Bacteriodes* spp. were grown in reinforced clostridia medium, the *Lactobacillus* spp. were grown in tomato juice broth, the *Escherichia coli* was grown in Luria-Bertani (LB) broth, the *Campylocacter jejuni* was grown in brucella broth, and the *Streptococcus pneumoniae* was grown in brain-heart infusion (BHI) broth supplemented with skim milk as the source of the complex media grown samples. The bacteria were grown in a semi-synthetic medium developed at Ross Laboratories (Columbus, OH) as noted. This medium consists of acetate and lactose as the primary carbon sources and ammonium chloride as the primary nitrogen source. The medium is also supplemented with various vitamins and trace elements.

The bacteria were grown in 10 ml cultures overnight (18-20 hrs.) under appropriate oxygen and temperature conditions for the strain. The bacteria from 1 ml of the culture were harvested by centrifugation and the growth medium discarded. The bacterial pellet was suspended in sterile distilled water and centrifuged again to remove traces of the growth medium. The supernatant was discarded and the bacterial pellet was resuspended in 1 ml of sterile distilled water. An aliquot was taken to perform viable counts. The serial dilutions were plated on a growth medium appropriate for the strain. The remainder of the solution was boiled for 15 minutes to kill the bacteria. The bacterial suspension was then dried under vacuum.

Media controls were prepared by diluting the grown medium 1:20 and then boiling the solution (1 ml) for 15 minutes. The

media solutions were dried under vacuum.

Bacterial Extract/Filtrate:

The bacterial culture was centrifuged to harvest bacteria, and the supernatant was filtered through a 0.22 μ membrane (Corning, New York, N.Y.) to obtain bacterial-free media in which bacteria was grown ("spent" medium). For further fractionation of molecules in "spent" medium, based upon their sizes, the "spent" medium was subjected to centrifugation in Microcon/Centricon filter cartridges from Amicon (Beverly, MA). The molecular weight cutoff (MWCO) for these membranes ranged from 3K (3 kilodaltons) to 100K (100 kilodaltons). The filter used for 1K MWCO was Pall Filtron (Northborough, MA). For dose response studies, cultures were harvested in a JA-18 rotor from Beckman (Palo Alto, CA) at 10,000 rpm, and the supernatant was passed through a tangential flow membrane with 1 K MWCO using Pellicon (Millipore, Bedford, MA). The filtrate was then lyophilized and tested in the assay.

B. Determination of Defensin Activity By Use of Madin-Darby Bovine Kidney (MDBK) Cells:

Cell Culture:

MDBK cells were obtained from the ATCC in Rockville, Maryland and were maintained in growth medium (Eagle's MEM with Earle's BSS, 10% FBS, 0.1 mM non-essential amino acids and no antibiotics). For stimulation experiments, cells were plated into 6 well tissue culture plates and maintained for one to two days in growth medium until cells were almost confluent. The medium was then changed to serum free epithelial cell growth medium (Clonetics, San Diego, CA), and

the test material was added to the dish. The medium was withdrawn, and the cells were rinsed with PBS 20 to 24 hours later. Total RNA was then isolated.

RNA Isolation:

5 Total RNA was isolated using a GlassMax RNA isolation kit or Trizol reagent (Gibco/BRL, Grand Island, New York) according to protocols supplied by the manufacturer (Gibco/BRL, Grand Island, New York). RNA was quantified by measuring the OD260 of each sample.

RNA-Polymerase Chain Reaction:

10 Total RNA was treated with DNase I prior to reverse transcription and PCR. The DNase was heat inactivated at 65 °C in the presence of EDTA for 10 minutes. Reverse
15 transcription and PCR were performed essentially as described for the GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA). Briefly, 600 ng of total RNA was primed with poly dT and reverse transcribed with Murine Leukemia Virus reverse transcriptase in a total volume of 40 uL at room temperature
20 for 10 minutes and then at 42 °C for an additional 15 minutes. The reverse transcriptase was heat activated at 99 °C for 5 minutes, and the reaction was chilled on ice for 5 minutes. This reverse transcription reaction was split in half; one portion was used for amplification of the target defensin RNA,
25 and the other was treated in parallel to determine the bovine actin RNA level (negative control). Additional reagents necessary for the PCR reaction, including appropriate synthetic DNA primers (i.e., LAP primers: 5' CTC TTC CTG GTC CTG TCT 3' & 5' CTT CTT TTA CTT CCT CCT GCA GCA 3'; Tubulin
30 primers: 5' GTT CCC AAA GAT GTC AAT GCT GCC 3'; 5' ATG CTG CAA

GGC TGA AAG GAA TGG 3'), were added after splitting the reverse transcriptase reactions to bring the reaction volumes to 100 uL. The reactions were then subjected to thermal cycling as follows: 95 °C for 1 minute, 52 °C for 1 minute, 72 °C for 1 minute (30 cycles), 72 °C for 15 minutes, and soaking at 4 °C. The expected 200 base pair beta-defensin PCR product was measured by electrophoresis or QPCR.

Capillary Electrophoresis:

PCR products were analyzed on an Applied Biosystems 270A-HT capillary electrophoresis instrument (Foster City, CA) using the DNA Fragment Analysis kit (Applied Biosystems, Foster City, CA). Analysis was performed using a 42 cm capillary tube and high accuracy DNA fragment analysis reagents. Data was quantified using a Rainin Dynamax system (Rainin, Emeryville, CA).

Product Capture for QPCR System: 10 uL of the final PCR mixture were combined with 15 uL of strepavidin bead slurry (Perkin Elmer, Foster City, CA), 21.5 uL ddH₂O, and 3.5 uL of 10x PCR buffer to yield a 50 uL binding reaction. The binding reactions were incubated at 55 °C for 15 minutes with occasional agitation. 1 mL of QPCR assay buffer was then added to the reaction. The entire mixture was transferred to a QPCR assay tube product. Quantity was measured with the QPCR instrument (Perkin Elmer).

Tables I, II and III below illustrate the results of the above samples with respect to defensin stimulatory activity.

TABLE IDEFENSIN STIMULATION BY VARIOUS TEST-SAMPLES

	SAMPLE	STIMULATION
	Lactobacillus reuteri (complex medium)	+
	Lactobacillus reuteri (semi-synthetic medium)	+
5	Bacteroides ovatus (complex medium)	-
	Bacteroides ovatus (semi-synthetic medium)	+
	Bacteroides fragilis (complex medium)	+
10	Bacteroides fragilis (semi-synthetic medium)	+
	Campylobacter jejuni (complex medium)	-
	Campylobacter medium control	-
15	Escherichia coli O111 (complex medium-aerobic)	-
	Escherichia coli O111 (complex medium-anaerobic)	-
	Escherichia medium control	-
	Streptococcus pneumoniae (complex medium)	-
20	Streptococcus meduim control	-
	Xylooligosaccharide (XOS)	-
	Lacto-N-fucopentose I (LNF I)	-
	Lacto-N-fucopentose II (LNF II)	-
25	3'-Sialyllactose	-
	6'-Sialyllactose	-
	Carboxymethyl cellulose	-
	Gum arabic	-
30	Guar gum	-
	Karaya gum	+
	Locust bean gum	-
	Yeast glucans	-
	Yeast autolysate	+
35	Casein hydrolysate	-
	Proteose peptone	-

Rice flour

-

Pea protein

-

Pea protein hydrolysate

-

TABLE II

Sample	Medium	Relative LAP Expression Level	Score
-LPS	----- -	100%	-----
+LPS	----- -	240%	-----
Lactobacillus acidophilus	Complex	446%	+
Lactobacillus acidophilus	Semi-syn	81%	-
Lactobacillus casei	Complex	179%	+/-
Lactobacillus casei	Semi-syn	98%	-
Bifidobacterium bifidum	Complex	323%	+
Bifidobacterium bifidum	Semi-syn	294%	+
Bifidobacterium medium control	Complex	N.D.	-
Lactobacillus medium control	Complex	N.D.	-
Medium control	Semi-syn	N.D.	-
Bifidobacterium breve	Semi-syn	N.D.	-
Bifidobacterium breve	Complex	N.D.	-
Bifidobacterium infantis	Semi-syn	N.D.	+/-
Bifidobacterium infantis	Complex	N.D.	+

TABLE III

Sample	Medium	Score
Bovine Lactoferrin	-----	-
Human Lactoferrin	-----	-
Soy Collagen	-----	-
Whey protein concentrate	-----	-
Lacto Pep G	-----	-
PP1610	-----	-
PP750	-----	-
Ardex	-----	-
LNnT	-----	-
Maltrin M100	-----	-
Bifidobacterium longum	Complex	-
Bifidobacterium longum	Semi-syn.	-
BSA	-----	-
Bovine K-casein	-----	-
Human K-casein	-----	-
Bovine Glyco-macropeptide	-----	-
Soy Flour	-----	-
Defatted Soy Flakes	-----	-
Soy Protein Hydrolysate	-----	-
FOS	-----	-
FOS-P	-----	-
TOS	-----	-

With respect to Tables II and III, stimulation of bovine beta-defensin mRNA was measured by comparing peak areas obtained by capillary electrophoresis of the PCR products resulting from LAP oligonucleotide primers (5' CTC TTC CTG GTC CTG TCT 3'; 5' CTT CTT TTA CTT CCT CCT CCT GCA GCA 3') and actin or tubulin oligonucleotide primers (5' GTT CCC AAA

GAT GTC AAT GCT GCC 3'; 5' ATG CTG CAA GGC TGA AAG GAA TGG
3'). Lipopolysaccharide (LPS) was used as a positive control
for stimulation of the beta-defensin mRNA in MDBK cells. The
results indicate that, under appropriate growth conditions,
Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus
5 reuteris, Bifidobacterium bifidum, and Bifidobacterium
infantis were stimulatory. Bacteroides ovatus, Kary gum and
yeast autolysate also had stimulatory activities. The
measured beta-defensin expression level was normalized to the
actin expression level. However, the sensitivity of
10 measurement of the PCR products was further improved by
replacing capillary electrophoresis with the QPCR instrument.
This instrument uses luminescence from a reporter adduct
compound present on one of the oligonucleotide primers used
for PCR amplification with each RNA.

15 To characterize the activities of the bacteria, further
fractionation of bacterial cultures was performed. The L.
acidophilus bacterial cultures were passed through a 0.22 u
filter to collect the filtrate or "spent" medium for testing
20 in the MDBK cell assay and further fractionation. The
"spent" media from L. acidophilus culture was further
fractionated using a 100K, 50K, 30K, 10K or 3K MWCO filter
membrane. The retentate and filtrate from each fractionation
were tested in the cell assay (see Figure 2). The
25 uninoculated media was used as a negative control (negative)
and B. infantis bacteria which showed activity in an earlier
assay was used as a positive control (Pos. Bifido. old). In
addition, yeast extract, yeast autolysate, and L-rhamnose
were tested in the same assay. The data indicated that the
30 defensin stimulatory activity was present in the filtrates of

L. acidophilus cultures even when 3K MWCO membrane was used for fractionation. These data suggested that the activity present in the "spent" medium of L. acidophilus is associated with a molecule less than 3 Kd in molecular weight.

5 The "spent" culture medium from B. infantis was centrifuged through a 3 K MWCO filter, and the filtrate (i.e., 3 Kd filtrate) showed stimulation of beta-defensin mRNA in MDBK cell assay when tested in triplicate (see Figure 3). Also, the filtrate recovered by using a 1 K MWCO filter
10 membrane from L. acidophilus "spent" medium (i.e., L. acidophilus 1Kd filtrate) exhibited stimulation of beta-defensin mRNA in the assay (see Figure 3).

15 After scale-up of the L. acidophilus 1 Kd filtrate using Pellicon, the lyophilized material was resuspended in 1 ml and tested in duplicate for stimulation of the defensin mRNA. The dose-dependence of stimulation was evident up to the highest concentration (6.6 mg/ml) of the filtrate tested (see
20 Figure 4).

EXAMPLE II

EFFECT OF L. ACIDOPHILUS ON IMMUNODEFICIENT MICE

25 The beige-athymic (*bg/bg-nu/nu*) mouse has dysfunctional phagocytic cells and lacks thymus-matured T cells. This mouse is susceptible to lethal candidiasis under gnotobiotic conditions. The isogenic *bg/bg-nu/+* mouse has functional T cell-mediated immunity and is resistant to lethal candidiasis under gnotobiotic conditions.

30 For purposes of the present experiment, *bg/bg-nu/nu* and

bg/bg-nu/+ germfree mice were fed *L. acidophilus* filtrate from 1 kilodalton MWCO membrane (LA-1KF) in their drinking water at a concentration of 8 mg/mL for 7 days prior to oral challenge with 1×10^7 CFU of viable *C. albicans*. The feeding of 8 mg/mL LA-1KF in the drinking water was continued throughout the experiment, and the mice were fed sterile rodent chow (Purina 5010C, Ralston Purina, St. Louis, Missouri) ad libitum. Mice consume approximately 5 mL/water/day, and each mouse weighed approximately 20 g, providing for a dose of 2 g LA-1KF/kg mouse/day. A control experiment was conducted concurrently with the LA-1KF treatment experiment, and it consisted of *bg/bg-nu/nu* and *bg/bg-nu/+* mice that were given LA-1KF in their water, but not orally challenged with *C. albicans* to assess effects of the LA-1KF feeding treatment on the mice. Another control group consisted of mice that received drinking water without LA-1KF, but they were orally challenged with *C. albicans*.

The mice were maintained in the experimental groups for 4 weeks, at which time, they were euthanized and evaluated for the effects of LA-1KF on mucosal and systemic candidiasis. Survival of the test and control mice at 2 and 4 weeks after oral challenge with *C. albicans* was assessed. Numbers of *C. albicans* colonizing the stomachs, small intestines, ceca and colons of the mice were determined by culturing dilutions of intestinal contents or feces on Sabouraud's medium. The number of *C. albicans* disseminated to the spleen, kidneys, and liver was measured by dilution of organ homogenates and culturing for viable *C. albicans*.

TABLE IV

Relationship Between LA-1KF Feeding and Numbers of Viable *C.*

albicans

Treatment group	Mean $\pm$ SEM log ₁₀ CFU <i>C. albicans</i> /g (dry wt) feces
<i>bg/bg-nu/nu</i>	9.8 $\pm$ 0.1
<i>bg/bg-nu/nu</i> + LA1-KF	8.1 $\pm$ 0.4 ^a
<i>bg/bg-nu/+</i>	8.6 $\pm$ 0.2
<i>bg/bg-nu/++</i> LA-1KF	8.7 $\pm$ 0.3

Results were averaged from 3 to 7 samples per group.

^aThe treatment group was significantly less than the control group by Student's t-test, $p < 0.001$.

- 10 The LA-1KF reduced the numbers of viable *C. albicans* in feces of the *bg/bg-nu/nu* mice at 1-2 weeks after oral challenge.

TABLE V
Relationship Between LA-1KF Feeding and *C. albicans* Growth in
Different Sections of the Gastrointestinal Tract

Mean $\pm$ SEM log₁₀ CFU *C. albicans*/g
(dry wt) contents

Treatment group	Stomach	S. Intestine	Cecum	Colon
<i>bg/bg-nu/nu</i>	8.9 $\pm$ 0.3	9.1 $\pm$ 0.2	9.4 $\pm$ 0.1	9.1 $\pm$ 0.1
Bg/bg-nu/nu + LA-1KF	7.9 $\pm$ 0.6 ^a	8.5 $\pm$ 0.1 ^b	8.7 $\pm$ 0.1 ^b	8.1 $\pm$ 0.7 ^c
<i>bg/bg-nu/+</i>	8.5 $\pm$ 0.2	8.1 $\pm$ 0.3	9.1 $\pm$ 0.2	8.3 $\pm$ 0.2
Bg/bg-nu/++ LA-1KF	7.4 $\pm$ 0.4 ^b	7.4 $\pm$ 0.4	8.8 $\pm$ 0.2	7.7 $\pm$ 0.5

All mice were fed 8.0 mg/ml LA-1KF p.o. in drinking water 1 week prior to and during colonization with *C. albicans*. Results were averaged from 3 to 5 samples per group. Results from *bg/bg-nu/nu* mice treated with LA-1KF were from 3 mice that were moribund at 2 wk after challenge. No *bg/bg-nu/nu* mice with candidiasis survived beyond 4 wk after challenge. LA-1KF-treated mice had significantly fewer *C. albicans* in the GI tract than untreated mice, as determined by Student's t-test, ^ap<0.01, ^bp<0.05, ^cp<0.001. Thus, in summary, LA-1KF feeding reduced the numbers of *C. albicans* in sections of the gastrointestinal tracts of *bg/bg-nu/nu* and *bg/bg-nu/+* mice 4 wk after colonization with *C. albicans*.

TABLE VI

Relationship Between LA-1KF and Dissemination of *C. albicans*

Treatment group	Dissemination ^a %	No. of <i>C. albicans</i> ^b
<i>bg/bg-nu/nu</i>	7/9 (78)	6.6 ± 6.5
<i>bg/bg-nu/nu</i> + LA-1KF:	3/3 (100)	4.7 ± 0.2 ^c
<i>bg/bg-nu/+</i>	4/11 (36)	7.7 ± 6.7
<i>bg/bg-nu/++</i> LA-1KF	1/5 (20)	3.8 ± 0

^aNo. of mice with disseminated candidiasis/no. of mice tested.^bNo. Of *C. albicans* = Mean + SEM log₁₀ CFU *C. albicans*/g homogenized tissues (1/3 spleen, 1/3 liver, 1/2 kidney combined).^cLA-1KF feeding significantly reduced the numbers of *C. albicans* in the spleen, liver and kidney, as compared to control mice, P<0.05 by Student's t-test.

LA-1KF treatment failed to reduce the incidence of *C. albicans* appeared to diminish the number of viable *C. albicans* isolated from internal organs. Further experiments will probably be necessary to conclusively determine the possible effect of LA-1KF feeding on the incidence of dissemination of *C. albicans* in this animal model.

TABLE VII

LA-1KF Feeding Induced Serum Antibody Production in *bg/bg-nu/+* Mice

Mean ± SEM ug/ml immunoglobulins

n=5 mice/group:

Microbial status	IgG	IgA	IgM
Germfree	102 ± 29	<200	26 ± 9
Germfree + LA-1KF	1451 ± 521	232 ± 32	205 ± 27
<i>C. albicans</i>	2257 ± 121 ^a	894 ± 21 ^a	54 ± 12
<i>C. albicans</i> + LA-1KF	2333 ± 481	991 ± 54	89 ± 11

* Not done due to early mortality of *bg/bg-nu/nu* mice colonized with *C. albicans*.

Based upon the results presented above, LA-1KF feeding significantly reduced the numbers of *C. albicans* in the spleens, kidneys and livers of the *bg/bg-nu/nu* mice. Also, numbers of *C. albicans* in the internal organs appeared to be decreased by LA-1KF feeding in *bg/bg-nu/+* mice. LA-1KF feeding also significantly reduced the numbers of viable *C. albicans* in the small and large intestines of the *bg/bg-nu/nu* mice compared with LA-1KF untreated, control mice.

Additionally, serum IgG, IgM and IgA production were increased after germfree *bg/bg-nu/+* mice were fed LA-1KF.

In conclusion, components of *L. acidophilus* present in the 1 kD LA-1KF preparation can mediate anti-infective and/or immunostimulatory effects on *C. albicans*-colonized mice. Such effects include reduction of *C. albicans* growth in internal organs and in the gastrointestinal tract, and induction of serum antibody production.

CLAIMS

1. A composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of a lysed yeast cell.

2. The composition of claim 1 wherein said portion is selected from the group consisting of cell wall components, cell surface components, cell membrane components, secreted products, the cell nucleus, and the endoplasmic reticulum.

3. The composition of claim 1 wherein said bacterial cell is of a genus selected from the group consisting of *Lactobacillus* and *Bifidobacterium*.

4. The composition of claim 3 wherein said bacterial cell is selected from the group consisting of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Lactobacillus casei*.

5. The composition of claim 1 wherein said yeast cell is of a genus selected from the group consisting of *Kluyveromyces*, *Saccharomyces* and *Hanensula*.

6. The composition of claim 5 wherein said yeast cell is selected from the group consisting of *Saccharomyces cerevisiae* and *Saccharomyces boulardii*.

7. The composition of claim 2 wherein said composition is a pharmaceutical composition or a nutritional composition.

8. The composition of claim 7 wherein said composition is in the form of a solid or a liquid.

9. A method of eliciting the production of defensins in eukaryotic cells comprising exposing said eukaryotic cells to a composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of a lysed yeast cell.

10. The composition of claim 9 wherein said portion is selected from the group consisting of cell wall components, cell surface components, cell membrane components, secreted products, the cell nucleus, and the endoplasmic reticulum.

11. The method of claim 10, wherein said bacterial cell is of a genus selected from the group consisting of *Lactobacillus* and *Bifidobacterium*.

12. The method of claim 11, wherein said bacterial cell is selected from the group consisting of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Lactobacillus casei*.

13. The method of claim 9, wherein said yeast cell is of a genus selected from the group consisting of *Kluyveromyces*, *Saccharomyces* and *Hanensula*.

14. The method of claim 13 wherein said yeast cell is selected from the group consisting of *Saccharomyces cerevisiae* and *Saccharomyces boulardii*.

15. The method of claim 9 wherein said eukaryotic cells are mammalian cells.

16. The method of claim 15 wherein said mammalian cells are epithelial cells.

5

17. The method of claim 16 wherein said epithelial cells are from a tissue or source selected from the group consisting of the tongue, the buccal mucosa, the nasal mucosa, the conjunctiva, the choroid plexus, the trachea, the bronchi, the lung, the fallopian tubes, the uterus, the cervix, the vagina, the uterus, the testes, the bladder, the urethra, the esophagus, the rumen, the reticulum, the omasum, the abomasum, the duodenum, the jejunum, the ileum, the caecum, the ascending colon, the spiral colon, the descending colon, the rectum, the brain, the kidney, the heart and the spleen.

10

15

18. A method of screening a test sample for defensin-inducing material comprising the steps of: 1) adding the sample to be tested for defensin-inducing activity to a cell line, 2) isolating total RNA from cells of said cell line, 3) quantifying said total RNA, 4) creating total cDNA by reverse transcription of said total RNA, 5) amplifying resulting beta-defensin cDNA, 6) analyzing amount of beta-defensin cDNA present in comparison to negative control cDNA, a higher amount of beta-defensin cDNA relative to control cDNA indicating a test sample which contains defensin-inducing material.

20

25

30 19. A method of identifying the presence of an up-

regulator of defensins in a test sample comprising the steps of: 1) constructing an expression vector comprising a defensin gene promoter operably linked to a reporter gene, 2) transfecting a host cell with said expression vector, 3) culturing said transfected host cell in the presence of said test sample, and 4) measuring the level of mRNA or reporter gene expression against a control, a higher level of mRNA or reporter gene expression relative to said control indicating the presence of an up-regulator in said test sample.

20. A method of treating or preventing a disease state in a patient in need of said treatment or prevention comprising administering to said patient a composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of a lysed yeast cell, in an amount sufficient to effect said treatment or prevention.

21. The method of claim 20 wherein said portion is selected from the group consisting of cell wall components, cell surface components, cell membrane components, secreted products, the cell nucleus, and the endoplasmic reticulum.

22. The method of claim 20 wherein said disease state is caused by a microorganism selected from the group consisting of a viral pathogen, a bacterial pathogen and a fungal pathogen.

23. The method of claim 20 wherein said bacterial cell is of a genus selected from the group consisting of

Lactobacillus and Bifidobacterium.

24. The method of claim 23 wherein said bacterial cell is selected from the group consisting of Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium infantis, and Lactobacillus casei.

25. The method of claim 20 wherein said yeast cell is of a genus selected from the group consisting of Kluyveromyces, Saccharomyces and Hanensula.

26. The method of claim 25 wherein said yeast cell is selected from the group consisting of Saccharomyces cerevisiae and Saccharomyces boulardii.

27. A vaccine comprising an adjuvant, wherein said adjuvant comprises an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of a lysed yeast cell.

28. A method of stimulating the immune system of a mammal comprising administering to said mammal a composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of a lysed yeast cell, in an amount sufficient to effect said stimulation.

29. The method of claim 28 wherein said portion is selected from the group consisting of cell wall components,

cell surface components, cell membrane components, secreted products, the cell nucleus, and the endoplasmic reticulum

30. The method of claim 29 wherein said bacterial cell is of a genus selected from the group consisting of
5 Lactobacillus and Bifidobacterium.

31. The method of claim 30 wherein said bacterial cell is selected from the group consisting of Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium infantis, and Lactobacillus casei.
10

32. The method of claim 29 wherein said yeast cell is of a genus selected from the group consisting of Kluyveromyces, Saccharomyces and Hanensula.
15

33. The method of claim 32 wherein said yeast cell is selected from the group consisting of Saccharomyces cerevisiae and Saccharomyces boulardii.
20

34. A rehydration composition comprising an extract derived from at least one member selected from the group consisting of a spent medium, a portion of a bacterial cell, a lysed yeast cell, and a portion of a lysed yeast cell.
25

35. A method of rehydrating a patient in need of such treatment comprising administering to said patient the rehydration composition of claim 33 in an amount sufficient to effect said treatment.

FIGURE 1

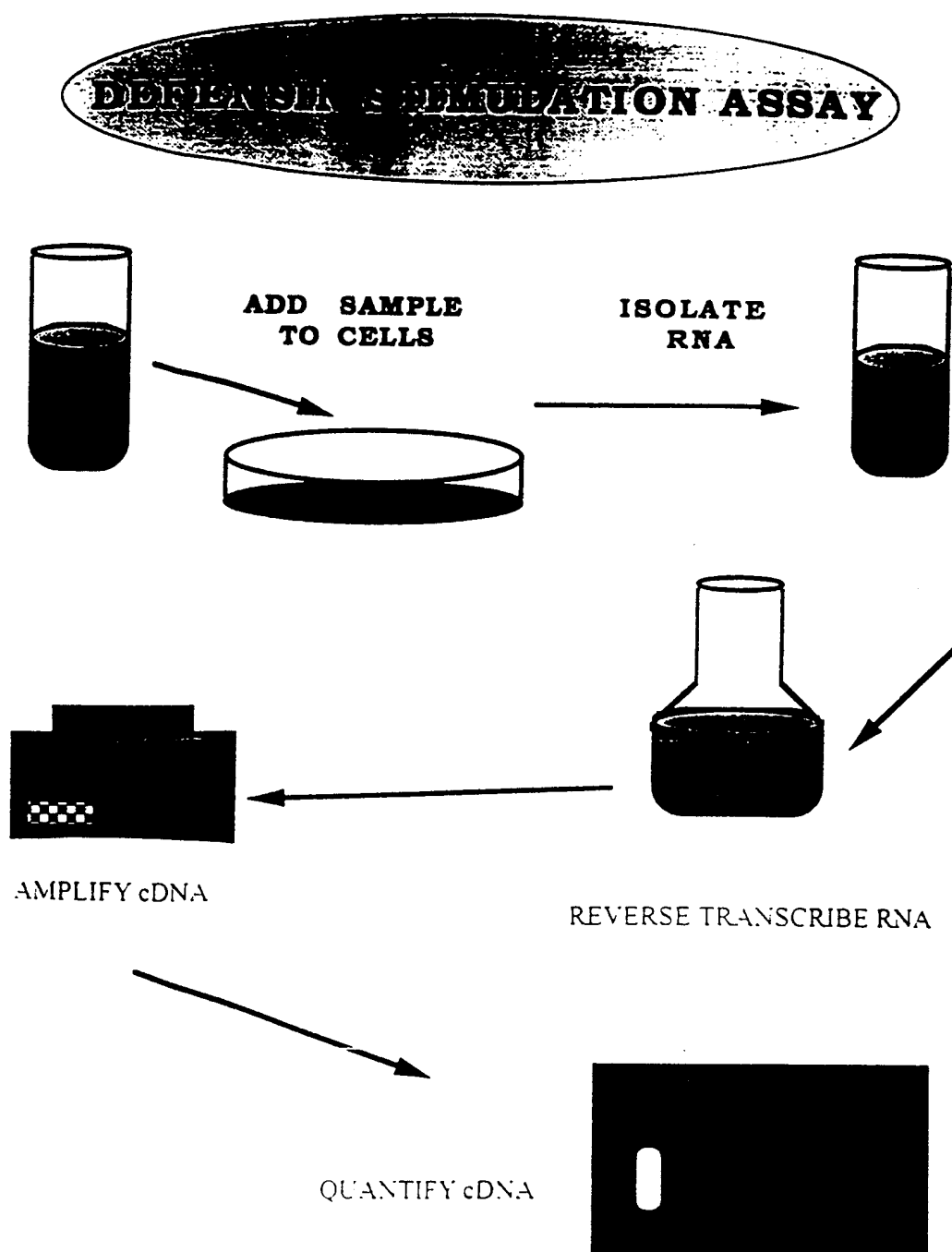


FIGURE 2

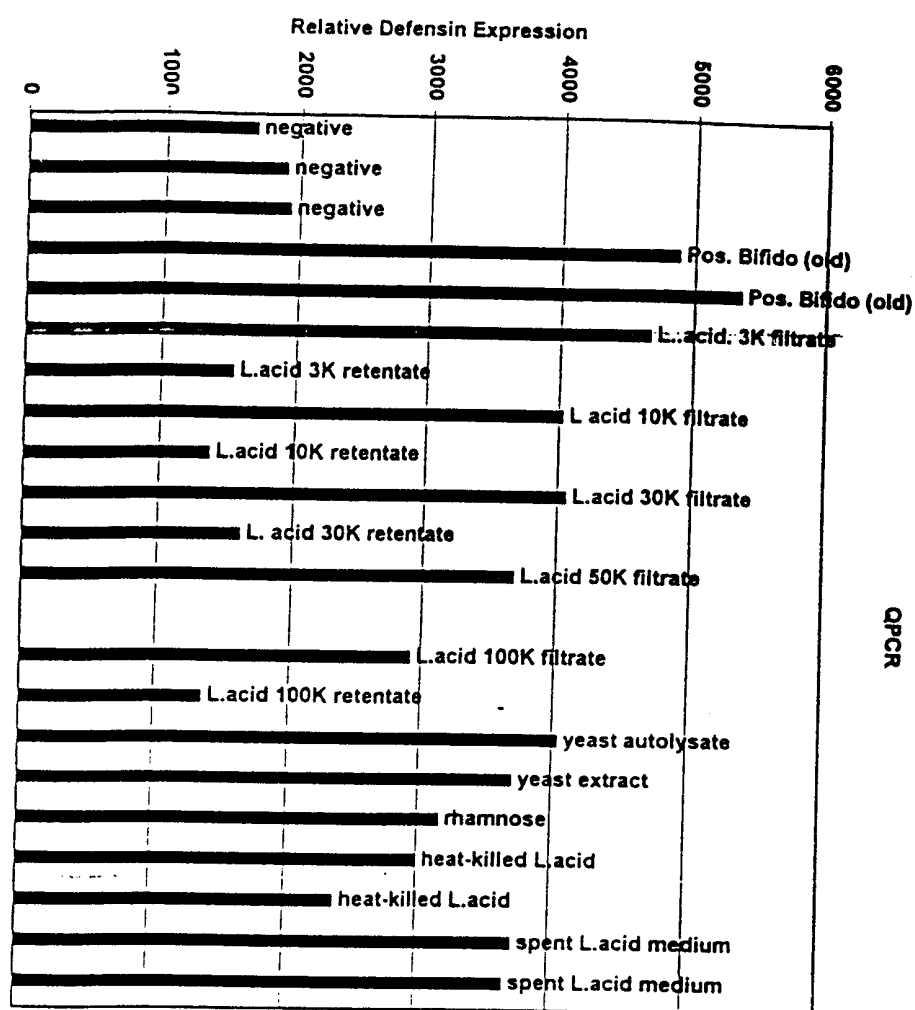


FIGURE 3

INDUCTION OF DEFENSIN EXPRESSION BY BACTERIAL FILTRATES

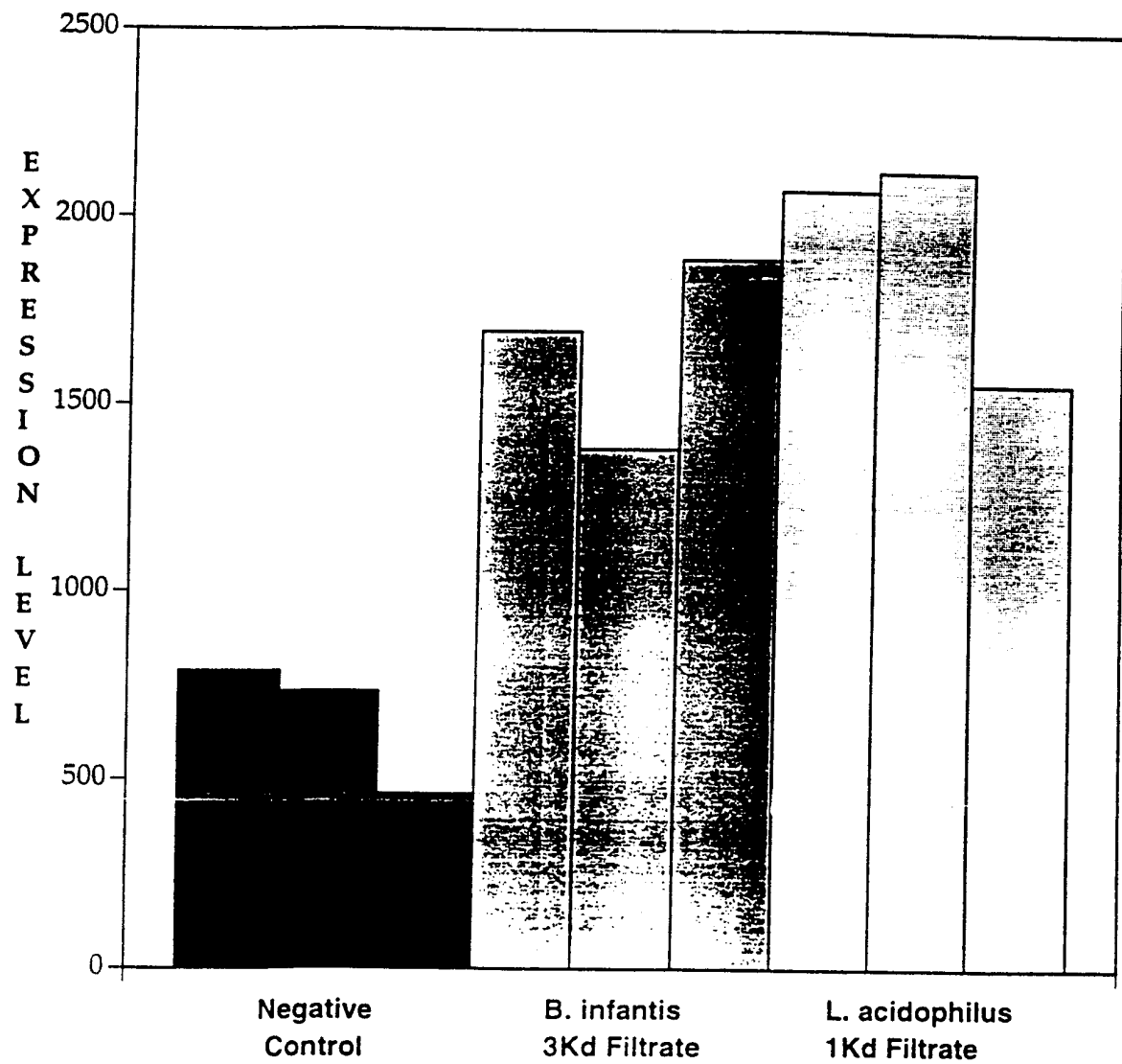


FIGURE 4

INDUCTION OF DEFENSIN EXPRESSION BY DIFFERENT CONCENTRATIONS
OF A 1KD FILTRATE FROM *LACTOBACILLUS ACIDOPHILUS*

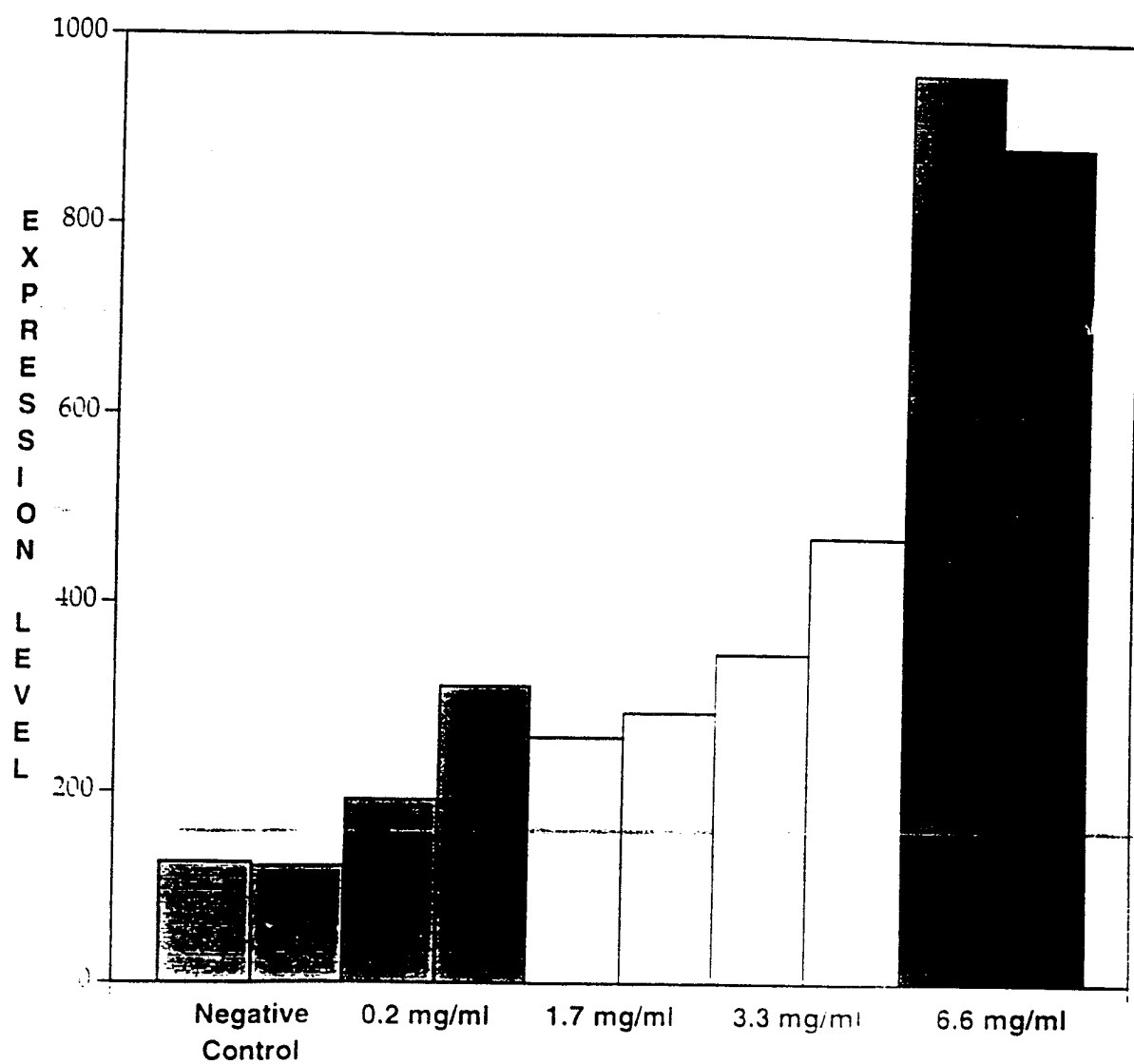


FIGURE 5

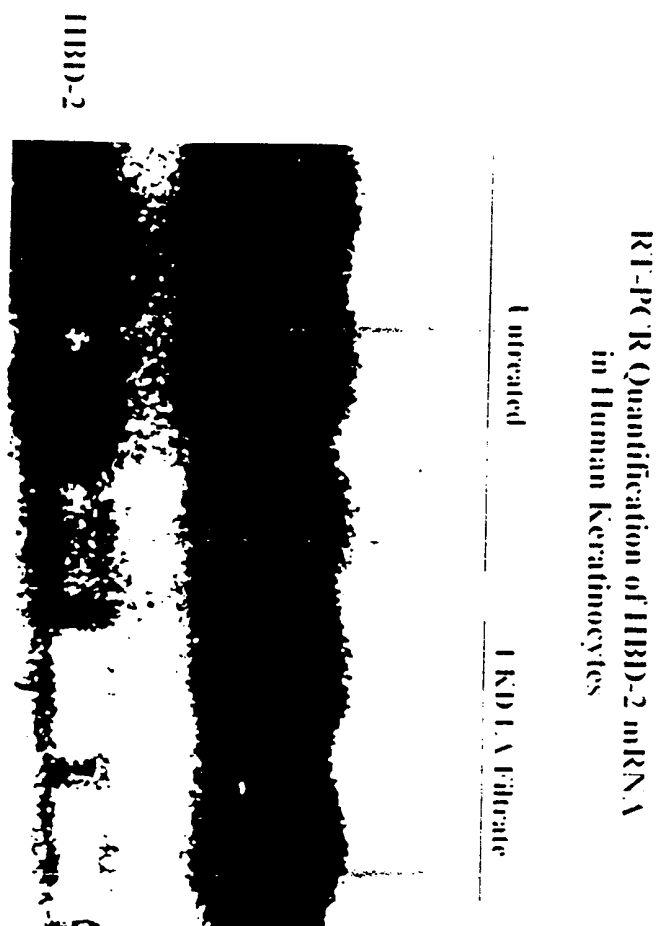
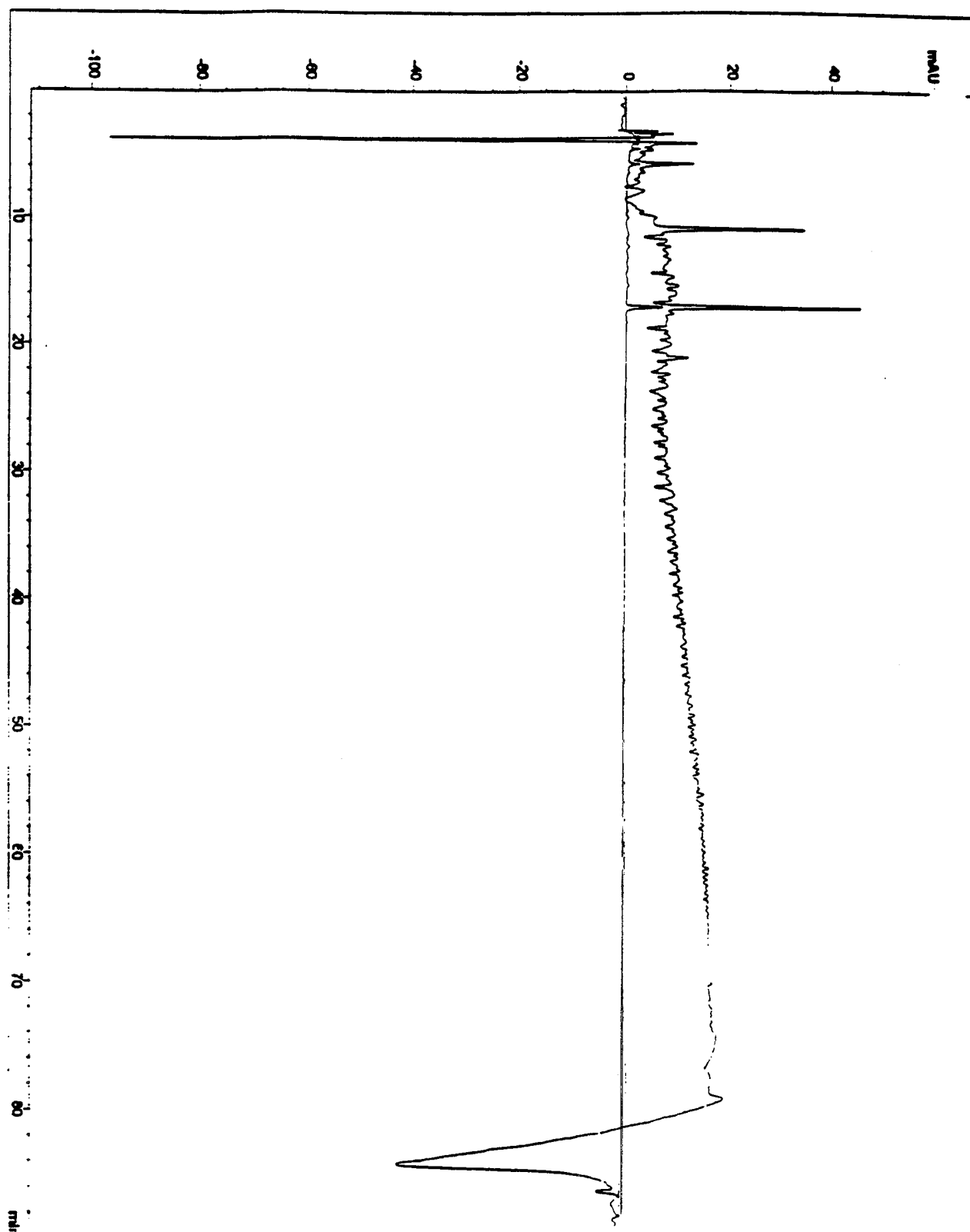


FIGURE 6



FIGURE 7



8/8

FIGURE 8

