'Role of Fructooligosaccharide, Buttermilk and Biogenic metabolites released from fermented beverage (Ambil) as a communicator between gut and brain'

Synopsis of PhD Thesis

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Introduction

Mental health statistics reflect the crux of integrated view of any society's health. However, mental illness still remains a taboo and is often underestimated. In recent years, over 90% of psychological counselling centre directors have reported significant increases in the number of college students with mental health problems out of which 70% people choose to suffer in silence rather than seeking professional help (Gallagher, 2011).

Two federal projects, the Surgeon General's Report on Mental Health (U.S. Public Health Service, 1999) and the President's New Freedom Commission (Hogan, 2003) on mental Health, have proclaimed stigma to be a ubiquitous barrier in enlisting professional assistance. Concerns were linked to reduced intentions to seek treatment among students who were asked to rate how likely they would seek help if they had suicidal thoughts (Yakunina et al., 2010). Mental viability is of utmost importance as it determines emotional, behavioural, and cognitive well-being, all of which contribute to maintaining a healthy balance of life activities and obligations while also promoting psychological fortitude so it should not be taken for granted. Anxiety disorder and depression are two of the most prevalent diagnostic groups that make up common mental diseases. Globally, more than 264 million people of all ages suffer from depression (WHO factsheet, 2020). It is anticipated to be the world's top cause of disability (James et al., 2020). Looking at the rise in trend of depression among youth, there is urgent need to address the problem by preserving and restoring mind's stability.

One probable solution lies in the highly dynamic human gastrointestinal tract. While billions of neurons are present in our brain, trillions of "good" bacteria exist in our intestines. These microbiotas play an important role in maintaining the functionality of the intestinal barrier which is critical for human's mental health (Kelly et al., 2015) making our gut popular as the 'second brain'. Microbes are thought to communicate directly with the brain through the circular communication loop involving central and the enteric nervous system, endocrine and immune signaling mechanisms amid the brain, gut, and gut microbiome labeled 'Gut-Brain axis' (De Palma et al.,2014). The activity of 10^{14} intestinal microbiomes aid in the maintenance of normal homeostasis and the synthesis of a diverse range of biological and neuroactive molecules such as gamma-Aminobutyric acid (GABA), norepinephrine, dopamine, and short-chain fatty acids, all of which have antidepressant properties. The gut contains 90% of the body's serotonin, which is a key regulator of gastrointestinal motility and the body's feel-good factor (Terry and Margolis2017). The vagus nerve is also important in mediating effects on the brain and behavior (Forsythe et al., 2014). A number of clinical findings link the changes in the gut microbial ecology to a variety of psychiatric diseases. The long-held belief that gut microbes interact with the central nervous system

(CNS) through influencing brain chemistry and affecting neuro-endocrine systems involved in stress response, anxiety, and memory function is the source of much of the enthusiasm around the human microbiome's ability to influence mental health.

Interestingly, biohacking our mental health is possible via the modulation of gut floras to a positive balance. Prebiotics, probiotics, antibiotics, and biogenic metabolites produced by milk fermentation open up new avenues for psychological therapies by altering the intestinal microbiota axis (Carabotti et al., 2015).

Fermentation has the potential to enhance the unique nutritional or phytonutrient content of foods, whose final worth is linked to mental wellness (Selhub et al., 2014). There is a strong link between the consumption of fermented beverages and the reduction of neurodegenerative diseases. Microbes influence the pre-consumption of food items, and these fermented dietary items, in turn, alter our microbiota. One probable reason for the psitive effect of fermented milk is the participation of biogenic metabolites, which are created by the action of microorganisms (Pessione and Cirrincione, 2016). The proteolytic system of *lactic acid bacteria* operates on casein found in milk and milk derivatives to provide bioactive peptides (Clare and Swaisgood; 2003). These peptides have been shown to improve brain cognitive performance by improving gut-brain connection (Dinan, and Cryan; 2017). Even though most shelf-available fermented beverages are tetra packed, we may still claim the products' health benefits since released biogenic metabolites make the product directly useful without the requirement for live bacteria (Korhonen and Pihlanto, 2003).

Buttermilk is one such fermented dairy products which has long been an important component of nutritional diet. Only a very limited number of scientific reports have been published on the use of buttermilk as a vehicle of probiotic cultures. However, it can be assumed that the buttermilk does contain several species of beneficial microorganisms. *Lactobacillus* and *Bifidobacterium* are the probiotic genera most widely used in the food industry. It is estimated that over 70 products containing *Lactobacillus acidophilus* and *Bifidobacterium* subsp., including yoghurt, buttermilk, frozen desserts and milk powder are produced worldwide (Shah 2007). Probiotic profiling of buttermilk revealed species isolated from Genus *Lactobacillus Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus casei and Lactobacillus paracasei*. Predominant species isolated from Genus *Bifidobacterium were Bifidobacterium bifidum, Bifidobacterium animalis, Bifidobacterium adolescentis* (Hirdyani and Sheth, 2018).

FOS has been recognised for its remarkable potential as a significant food component in enhancing bacterial bionomics, which has shown a direct role in reducing the burden of diseases like CVDs improving glycaemic, lipemic control and reducing the expression of inflammatory markers (Ooi and Liong, 2010, Dehghan et al 2014, McLoughlin et al., 2017). FOS consumption has

also been linked to significant reductions in TG, TC, LDL-C, VLDL, and HDL-C, as well as an increase in HDL-C. (Sheth et al, 2015). Many studies have also observed significant reductions in hs-CRP levels and BMI (Sheth and Asudani, 2014; Jain and Sheth 2014). The ability of prebiotics to regulate the dysbiosis microbiota associated with psychological illnesses such as anxiety, depression, stress, autism, learning, and memory has also gained traction (Clapp et al. 2017). Seeing the health benefits of this prebiotic, it can undoubtedly be claimed that FOS holds great potential in nutraceutical industry. Feeding the right gut bacteria for the brain can be the solution to combat mild to moderate depression.

Rationale

It is evident that a combination of prebiotic added fermented beverage which may have biogenic metabolites in sufficient amounts would trigger the colonization of good bacteria in the gut and thus activate the channels of gut brain connection to bring about an improvement in the depression status of the individuals. Fermented beverage, prebiotics and probiotics carries significant implications for research that points the way toward dietary interventions to improve brain function. These foods are a good remedy for anxiety and depression and are safer than conventional medication besides providing additional health benefits. The discovery that changing the bacterial environment in the gut can affect the brain has long been surmised in animal data and it looks interesting and suggestive, but still, there is no convincing human study. So, this research was being undertaken to 'Quantify the presence of biogenic metabolites in prebiotic enriched milk based fermented beverage (ambil), and to measure if the consumption of ambil, prebiotic (fructooligosaccharide) and buttermilk can associate between gut microbiota and emotional psychiatry, ruling out the most effective intervention among the following in lowering mild to moderate depression.

Specific Objectives

1) To determine biogenic metabolites in prebiotic enriched milk-based fermented beverages (casomorphin and casoxin).
2) Assessing depression levels of students of faculty of Family and Community Sciences aged 19-30 years using Beck's Depression Inventory.
3) To randomly classify subjects who are mild- moderately depressed and willing to participate in the study into 5 groups (experimental group1, experimental group 2,

experimental group 3, experimental group 4, and control group) and collect baseline data regarding their general information, medical history, personal habits and study their biophysical profile in terms of gut microflora (*Bifidobacteria, Lactobacillus*, and *Enteropathogenic* bacteria) and cortisol levels (a biomarker of stress).

4) To conduct intervention trials with four different products viz.200ml Ambil, 10 ml FOS, 200ml fresh buttermilk, and 200 ml buttermilk tetra pack in the routine food item, for 45 days.

5) Post- data collection on the parameters similar to baseline.

6) To develop IEC material to combat mild to moderate levels of depression.

Hypothesis

Null Hypothesis

- There is no association between consumption of fructooligosaccharide, buttermilk and prebiotic enriched cereal buttermilk based fermented beverage (ambil) with lowering depression scores, cortisol levels and improvement in gut microflora.

Alternate Hypothesis

- There is association between consumption of fructooligosaccharide, buttermilk and prebiotic enriched cereal buttermilk based fermented beverage (ambil) with lowering depression scores, cortisol levels and improvement in gut microflora.

Review of Literature

Following flow was followed to read the collected literature about the present research:

- ➢ Incidence and prevalence of mental disorders

- ➢ Incidence and prevalence of depression
    - o The burden of depression across the states of India
    - o Gender wise prevalence of depression
- ➢ History of depression
- ➢ Symptoms of depression
- ➢ Depression and its types
- ➢ Measuring depression
    - o Inventory scales
    - o Biomarkers for depression assessment
- ➢ Unmasking the Invisible World: The Microbiome
    - o Glimpse at human gut microbiota
    - o Shaping of human gut through life span
- ➢ Leaky gut leads to brain functional disorientation
- ➢ Gut -Brain axis
- ➢ Medium of crosstalk between microbiota–gut–brain axis
    - o Vagus nerve
    - o Neuroendocrine: hypothalamic–pituitary–adrenal axis
    - o Neuropeptides
    - o Short chain fatty acid
    - o Neurotransmitters
- ➢ Proven strategies for microbial modulation

- ➢ Fecal microbiota transplantation
  - o Probiotics
  - o Prebiotics
- ➢ Fructooligosaccharide as a prebiotic
  - o FOS safety, tolerance and caloric value
- ➢ Implication of FOS on gut and brain
- ➢ Fermentation: an age-old miracle invention
  - o fermentation process
  - o functional properties of fermented foods
- ➢ The role of biologically active peptides derived from fermented milk on brain's health
- ➢ Mechanism of action of psychobiotics

Methodology

This research study was divided into 8 phases.

PHASE -I: *Quantification of Biogenic metabolites (B Casomorphin and Casoxin c) in ambil using High Performance Liquid Chromatography.*

PHASE -II: *Snap -Shoting the presence of mild to moderate depression among the subjects in the age group of 19-30 in the Faculty of Family and Community Sciences, The Maharaja Sayajirao university of Baroda, Vadodara*

- ▣ Screening of the subjects from the Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda for mild to moderate depression using Beck's Depression Inventory.
- ▣ Collection of baseline data of subjects enrolled regarding their general information.
- ▣ Assessing the macro and micro nutrient intakes, and frequency of consumption of probiotic and prebiotic foods of normal, mild to moderate and severe depressed subjects.
- ▣ Mapping the morbidity profile, defecation pattern, personal habits as reported by normal, mild to moderate and severe depressed subjects.
- ▣ Assessing correlation among various parameters mentioned above with the fecal microbiota count and blood serum cortisol levels of mild to moderately depressed subjects.

PHASE -III: *Impact evaluation of intervention trials with ambil (prebiotic enriched fermented beverage) on the fecal microbial counts (Lactobacillus, Bifidobacteria and Enteric pathogens), depression status, serum cortisol and defecation profile of mild to moderately depressed subjects.*

PHASE -IV: *Studying the prebiotic efficacy of FOS supplementation by determining fecal microbial counts (Lactobacillus, Bifidobacteria and E. coli), depression status, serum cortisol and defecation profile of mild to moderately depressed subjects before and after supplementation.*

PHASE -V: *Pre and post assessment of gut microbiome log count with respect to Lactobacillus, Bifidobacteria* and *E. coli, state of depression, blood serum cortisol and degree*

of constipation in mild to moderately depressed subjects after intervention with tetra packed buttermilk.

PHASE -VI: Assessing the potential of fresh buttermilk in modulating the gut floras (Lactobacillus, Bifidobacteria and E. coli), checking depression, blood serum cortisol and defecation profile in mild to moderately depressed subjects.

PHASE -VII: Evaluating which intervention had highest impact in reversing depression scores, cortisol levels and modulating gut microbiota composition.

PHASE -VIII: Developing an audio-visual animated aid as a means of IEC material to make general people aware about increasing trend of depression and how to cope up with it.

4.1 Quantification of biogenic metabolites from Fructooligosaccharide enriched buttermilk based fermented beverage

Biogenic metabolites viz. Casoxin c and ß Casomorphin were quantified from ambil (Fructooligosaccharide enriched buttermilk based fermented beverage) using high pressure liquid chromatography (HPLC) by means of Evaporative Light Scattering Detector. Column used was Lichrospher, and the temperature was maintained at 32° C. Standards for ß Casomorphin and Casoxin C was purchased from Sigma Aldrich.

1

Preparation of mobile phase: buffer solution was prepared by mixing HPLC grade acetonitrile and water in the ratio 82:18.

2

Standard solution- standards of ß Casomorphin and Casoxin C 102.25mg each were dissolved in two separate volumetric flask of 50 ml containing mobile phase maintaining the temperature 20°C.

3

Sample solution - Ambil 200ml was poured in rotary flask and allowed to stay in vacuum condition (105°C for 2 hours). Dried sample (100mg) was then dissolved in 50 ml volumetric flask containing mobile phase (buffer solution: HPLC grade acetonitrile and water 82:18 at 20°C)

Calculating the percentage of ß Casomorphin and Casoxin C: Flow rate was adjusted to separate the standards. The instrument was equilibrated by pumping mobile phase through it until a stable baseline was obtained. Peak areas of the sample solution were measured. Chromatograms of the standard and sample solution were recorded.

Percentage of casoxin C and ß casomorphin was calculated from the formula:

Weight of standard/ weight of sample x peak area of sample/ peak area of standard x factor

4.2 Location of the study

Department of Clothing and Textiles, Department of Extension and Communication, Department of Family and Community Resource Management, Department of Food and Nutrition, Department of Human Development and Family Studies in the vicinity of The Faculty of Family and Community Sciences, The MS University of Baroda, Gujarat were purposively selected for carrying out the current research. Written permission was sought from the head of respective departments within faculty and from the psychologist, MARG Health center, The MS University of Baroda before initiating the screening process.

4.3 Screening of the subjects for depression

Subjects aged 19-30 years who gave the consent to participate were screened using a purposive sampling procedure from The Faculty of Family and Community Sciences, The MS University of Baroda, Gujarat. Beck's Depression Inventory was administered to 683 subjects to determine the presence of depression, and subjects were classified into normal (Score 0-11), mild (score 11-16), borderline clinical (score 17-20), moderate (score 21-30) and severe category (score >31) based on the answers marked. Participants were oriented regarding the objective and the implications of the study before further assessment.

4.4 Survey

To obtain a better picture about the subjects enrolled, a questionnaire-based survey was conducted using a self-assessed questionnaire which gathered background information on the following aspects Date of birth and gender, Type of family and Total family members, Total family income, Education, Past medical history, medication and hospitalization in last six months.

4.5 Dietary intake

Diet determines different aspects of one's health. 24 hours diet recall for three days was taken from all the enrolled subjects through self-administered questionnaire. Details of all the meals consumed throughout the day was taken into account. Total energy intake, nutrient components such as carbohydrates, protein, fat, iron, calcium, zinc, vitamin C and non-nutrient component such as total dietary fiber were calculated. The cooked value of the food intake of each meal was priorly converted into raw values and then entered in Diet Soft Software [Kaur, 2007] to obtain the nutrient intake of each individual. Frequency of consumption of Prebiotic, probiotic and fermented beverages like buttermilk, kadhi, chaach, curd, shrikhand was also noted and recorded using modified food frequency questionnaire (FFQ). Details of the number of times the food items were consumed on a daily, weekly, monthly, or yearly basis was elicited using the Food frequency questionnaire.

4.6 Inclusion and exclusion criteria to be part of the experimental groups

To be the part of intervention trials subject must belong to the age group between 19-30, must score between 11-30 on the Beck's Depression Inventory scale which underline them in the mild to moderate depression category. Should give written consent to participate in the research at their own will. Subjects showing medical history and on medication for any of the chronic disease like diabetes, hypertension, thyroid, cardiovascular disease, neurological deformities, renal or liver disorders, cancer, any type of infections or hospitalization in last three weeks were excluded from participating in the research. Those who fell in the category of severe depression were asked to seek expert help and given reference of the psychologist, Health center, The MS University of Baroda.

4.7 Study design for randomized clinical trial

Beck's Depression Inventory was introduced to assess the levels of depression in subjects aged 19-30 from the various departments of Faculty of Family and Community Sciences. Total screened subjects were 683. Subjects who scored between 11-30 on inventory were categorized under mild to moderate depression category (n=368). Using systemized random sampling these subjects were further segregated into five groups of 30 individuals each viz. experimental group 1, experimental group 2, experimental group 3, and experimental group 4 to which ambil, FOS, tetrapacked buttermilk and fresh buttermilk were intervened respectively. No intervention was given to control group. After supplementation period of 45 days, impact evaluation of various food products was analysed with respect to the consumers depression status, fecal microbial

log count, blood serum cortisol and defecation profile in terms of stool frequency.
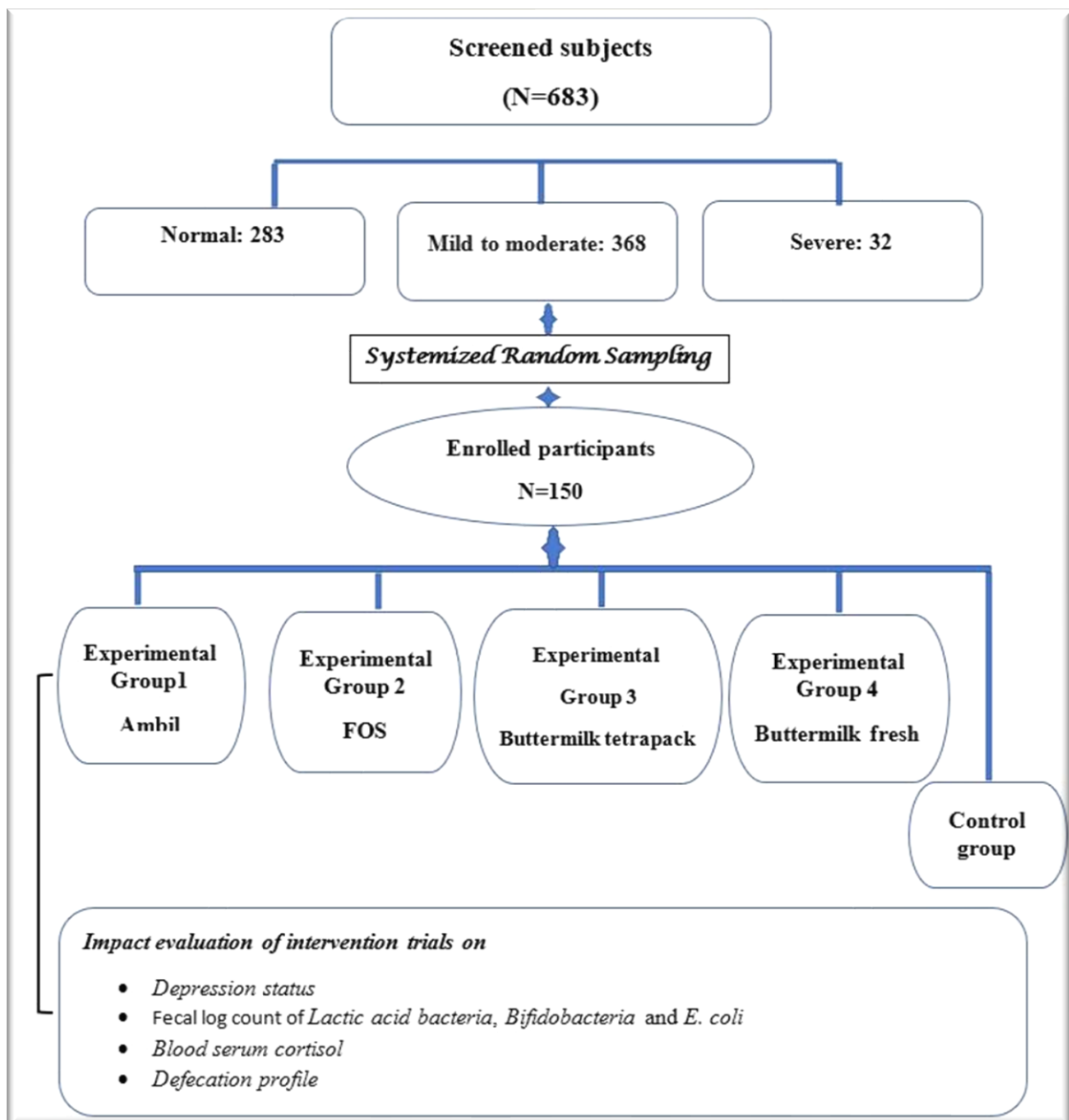

Figure 5.1 Experimental design for randomized control trial

4.8 Procurement of the intervention supplements

Ambil

Ambil used for supplementation was made up using rice powder and buttermilk enriched with fructooligosaccharide following standardized recipe (Ramakrishnan 1977; 1979) by Hirdyani and Sheth, 2018.

Fructooligosaccharide

Liquid fructooligosaccharide (25 litres bottle; Type L95; Batch No. FL915071-T) was procured from Tata Chemicals Limited - Innovation Centre, Mumbai. The fructooligosaccharide was food grade which can be used as fat and sugar replacer. The specifications of the fructooligosaccharide used for intervention are given in Table 5.2

Table 5.2: Fructooligosaccharide specifications

Items		Product Code - Fossence(L-95)
		Specifications
Sensory	Appearance	Clear to light yellow translucent and sticky liquid
	Insoluble	No visible insoluble
	Smell	Special FOS smell
	Taste	Good sweet taste and no odor
Physicochemcial Index	Dry matter (Solid material) % $\geq$	75
	pH value	5.5~7.5
	Total FOS (on dry matter basis) (%)$\geq$	55
	Ash(%)$\leq$	0.4
	Transmittance(%)$\geq$	85
Hygienic Index	Arsenic(mg/kg) $\leq$	1.0
	Lead(mg/kg) $\leq$	5
	Total bacteria number(cfu/g) $\leq$	1000
	Coliforms （MPN/100g） $\leq$	30
	Pathogenetic germs (Salmonella, Shigella, Staphylococcus)	None

Buttermilk

Buttermilk 200ml tetrapackets were procured from Amul, Gandhinagar. Fresh buttermilk was obtained from Paras, Vadodara Dairy Baroda.

4.9. Determination of the Gut Microbiota

The gut microbiota was determined with respect to fecal- *Lactic acid bacteria, Bifidobacteria* and *E. coli.*

The steps involved in the determination of the fecal flora were:

4.9.1. Collection and Storage of the fecal sample

4.9.2. Sterilization of the glass wares

4.9.3 Preparation and Sterilization of dilution blanks

4.9.4 Preparation and Sterilization of Media

4.9.5 Preparation and inoculation of sample

4.9.1 Collection and Storage of the fecal sample

The subjects were given air tight sterile clinicols which were labelled properly to avoid inconvenience, for collection of their stool samples. They were asked to bring their stool samples just before coming to department. Samples collected were immediately stored at appropriate temperature (-20°C) in deep freezer and were analyzed within 2 days after collection.

4.9.2 Preparation of the sample

One gram of faecal sample was weighed on a sterilized balance and was used for bacterial enumeration.

4.9.3 Sterilization of the glass wares

All the petri dishes and the other glass wares such as beakers and conical flasks were sterilized before use. The petri dishes were kept in the petri dish box and the other glassware's were kept in a hot air oven at 180^0C for 2 hours for sterilization. The micro-tips were decontaminated by autoclaving at 121^0C for 15 minutes at 15 lbs. pressure. The other instruments which were used like the weighing balance and spatula were all sterilized by alcohol swabs and alcohol flaming using 70 % alcohol. Sterile petri-plates were used and opened under the laminar only at the time of plating.

4.9.4 Preparation and Sterilization of dilution blanks:

For the preparation of dilution blanks 1 g of peptone was dissolved in 1000 ml of distilled water. This solution was transferred in portion of 100 ml in 10 dilution bottles. These bottles were autoclaved at 121^0C for 15 minutes and cooled at room temperature before putting them to use [IGNOU, 2005].

4.9.5 Preparation and Sterilization of media

The media used for the enumeration of *Bifidobacteria* was Bifidobacterium agar, MRS Lactic acid bacillus agar and Violet Red Bile Agar was used for *Lactic acid bacteria* and *E. coli* respectively. Media were supplied by Hi. Media. The prepared media of Bifidobacteria and LAB were autoclaved at 121ºC for 15 minutes whilst EMB agar was prepared just before pouring. The prepared media were then poured into sterile petri-plates and allowed to set inside the laminar flow under UV light. Media used for different gut flora is presented in Table

Table 5.3: Details of media used in determining the gut flora subjects

Gut Flora	Incubation Condition	Incubation Duration	Temperature	Media Used (Brand)
Bifidobacteria	Inside anaerobic jar with an anaerobic	48 hours	37ºC	Bifidobacterium Agar (Hi. Media®)
Lactic acid bacteria	Inside dessicator with calcium	48 hours	37ºC	MRS Lactic acid bacillus agar (Hi. Media®)
E.coli	Inside incubator	24 hours	37ºC	Violet Red Bile Agar (Hi. Media®)

4.9.6 Preparation and inoculation of sample

Frozen fecal sample was first thawed and brought to room temperature; one gram of fecal sample was accurately weighed and mixed homogeneously in 99 ml of 0.1% peptone water to provide 1% (w/v) fecal slurry. One ml of slurry was diluted serially in peptone water using stomacher and 0.1 ml of sample was pipetted from each of the dilutions to the petri plates containing respective media. The above procedure was carried out inside laminar flow that ensured a sterile environment thereby preventing contamination from outside.

4.9.7 Incubation and enumeration of Lactic acid bacteria

Petri plates of Lactic acid bacteria were placed in a dessicator as it is a facultative anaerobe, and the dessicator were then placed in the incubator at 37ºC for 48 hours of incubation.

4.9.8. Incubation and enumeration of Bifidobacteria

The plates of Bifidobacteria were incubated at 37ºC placed in the anaerobic jars using anaerobic gas-packs procured from Hi. Media® for 48 hours.

4.9.9 Incubation and enumeration of E. coli

Petri plates of E. coli were directly placed in the incubator at 37ºC for 24 hours.

4.9.10 Colony counting

After completion of incubation period (Plate 4.3) of the respective bacteria, petri plates were then placed on colony counter. The colonies that appeared in the range of 30 – 300 were converted in to log counts after multiplying with their dilution factors [Ramona et al., 2000] and further used for statistical analysis

4.10. Assessment of cortisol levels

Cortisol levels were determined in blood serum, by an expert lab technician.

- The flow of blood in the arm was stopped by wrapping an elastic band around the upper arm, which made the veins in arm to become more visible and easier to insert the needle.

- Alcohol was used to clean the site on the skin where the needle was inserted. The needle was inserted into the vein, and blood was collected in a tube that's attached to the needle.

- After the needle was removed from the skin, cotton or gauze was placed on the site of the needle insertion. A bandage was used to secure the cotton or gauze.

- Blood was drawn into plain tubes, preserved freezed at $-4°C$ and then separated for subsequent measurement of cortisol in serum with the IMMULITE. The volume of serum required was 10 µl. Cortisol was determined at pharmacological lab from blood serum by Chemi Luminescent Immuno assay in ROCHE machine.

4.11. Supplement monitoring and data compliance

Participants of experimental group 1 and 3 received the supply of ambil and buttermilk respectively every week consecutively for 6 weeks in a row. Subjects falling in experimental group 2, were provided with liquid FOS in a 500 ml food grade plastic container. Subjects who were intervened with fresh buttermilk were asked to collect buttermilk daily from the mess, where the record book was maintained. All the participants were asked to send daily picture of themselves consuming the product and of the empty cartoon post intake.

Compliance was monitored with appropriate follow ups using daily SMS reminders and phone calls fortnightly. A calendar was especially designed and distributed to all the participants to

document daily consumption of supplements and any unusual symptoms or side effects if observed. Calendars were collected back after the completion of intervention period.

4.12. Post data collection

Post data was collected on the parameters similar to the baseline i.e., Beck's Depression Inventory, serum cortisol, defecation profile and gut microflora (*Lactobacillus, Bifidobacteria* and *E. coli)*

4.13. Statistical Analysis

To provide an evidence of significance of the data observed during the course of research, statistical analysis is an inevitable tool. Present research was validated using below stated analytical software (Table 4.2). The data was entered in an excel spreadsheet (MS, 2007). The data was cleaned, verified, and subjected to appropriate statistical analysis using Statistical program for social sciences (Trial version 20.0).

4.13.1. Chi Squared [χ2] Test

Chi-square is a versatile statistical test used to examine the significance of relationships between two (or more) nominal-level variables. It is a non-parametric test of significance and therefore used for all the qualitative data in the present research. The significance levels were set at 95% by two sided tests. Chi square, in the present research was utilized to determine the significance of difference based on gender, age, religion, education, family type and income and to determine the significance of difference in the incidence of stool frequency in different categories of depression.

4.13.2. Student's 't' test

Student's t-test is applied in order to find out the significance of difference in parameters of two different groups. The significance levels were set at 95% by two sided tests. Independent t' test is used in the present research for:

Determining the significance of difference in the mean counts of depression scores, serum cortisol, gut microflora, and defecation profile of subjects in experimental groups and control group.

4.13.3. Analysis of Variance (ANOVA) or 'F' Test

Analysis of variance (ANOVA) is a collection of statistical models used in order to analyse the

differences between group means and their associated procedures (such as "variation" among and between groups), developed by R. A. Fisher. In its simplest form, ANOVA provides a statistical test of whether or not the means of several groups are equal, and therefore generalizes the t-test to more than two groups.

4.13.4. Post Hoc LSD test or Fisher's Least Significant Difference (LSD)

Post hoc LSD was applied in the present research to ⌉ Determine the significance of multiple intervention on depression scores, serum cortisol, gut microflora, and defecation profile and to rule out which intervention was most effective.

4.13.5. Paired 't' test

A paired t-test measures whether means from a within-subjects test group vary over two test conditions. The paired t-test is commonly used to compare a sample group's scores before and after an intervention. The phases of the study involve analysing the impact of Ambil, FOS and buttermilk to mild to moderately depressed subjects for which this test was applied and the significance levels were set at 5% by two sided tests in order to:
 Determine the significance of difference obtained before and after the supplementation in the depression scores, serum cortisol, gut microflora, and defecation profile.

4.13.6. Pearson's correlation

Test was carried out in order to determine if there exist an association between grades of depression, cortisol levels, nutritional status, gut microbiota and defecation profile of subjects enrolled in the research.

4.14. Development of IEC material

Animated movie was developed using the software *Animaker*. Movie script was written keeping in mind the need to create awareness among general population about depression and how to overcome it. Different characters were allotted and dialogues were recorded and synchronized with particular scenes. The movie is named 'RESTART' which aim to ring the wake-up call to restart with new hope, new strength and new thought to overcome depression.

4.15. Ethical Committee Approval and Study Registration

The study protocol was approved by the Medical Ethics committee of the Foods and Nutrition Department, The M.S. University of Baroda– "Institutional Ethical Committee for Human Trials" in compliance with the guidelines issued by Indian Council of Medical Research with the medical ethics approval number (IECHR/2018/16). Written consent was obtained from the subjects participating in the study.

Results

Based on the objectives of the study, the outcome of the findings is presented in the eight phases in summarized format below:

PHASE -I: Quantification of Biogenic metabolites (B Casomorphin and Casoxin c) in ambil using High Performance Liquid Chromatography.

Percentage of casoxin C and ß casomorphin in ambil was calculated using the formula: Weight of standard/ weight of sample x peak area of sample/ peak area of standard x factor. As projected on HPLC graph, retention time for standards of casoxin *C and beta casomorphin* was 15.24 and for samples was 21.03. Percentage area covered by standards of *casoxin C* and *beta casomorphin* was 54.43% and 32.32% respectively. Percentage area covered by these biogenic metabolites in analysed fermented beverage was 27.96 % and 17.60 % respectively. The amount of casoxin C reported to be 21.3 mg/ kg and ß casomorphin to be 6.2mg/kg in ambil.

PHASE II: Snap -Shoting the presence of mild to moderate depression among the subjects in the age group of 19-30 in the Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda, Vadodara.

This phase of the study was planned to study the dominant status of depression among the subjects aged 19-30 years. Attempt was made to determine the role of various contributory factors which included general characteristics, medical history, dietary patterns, defecation profile, food and exercise habits. High percentage (53.8%) of university students scored between 11 to 30 on Beck's depression Inventory which put them under the category of mild to moderately depressed whereas, 32 students (4.68%) reported to suffer from severe depression as they obtained scores between 31 and 40 and less than 50% out of total screened i.e., 283(41.43%) scored normal score (0-10). Out of the total participants screened (n=683), 94.87 % were in the age group of 19-25 of which 41.20% scored normal, 53.85% were falling in the category of mild to moderate depression while severe depression was noted in 4.93% based on scoring pattern of Beck's depression inventory.

Moving forward with percentage of gender integrities, almost all the participants (98.6%) were females out of which 53.5% reported to be mild to moderately depressed. Screening for depression was carried on, 64.86% hindus, 32.94% muslims and 2.19% christians of which

50.11%, 60.88% and 60%, hindus, muslims and christians respectively reported to be mild to moderately depressed. Out of total mild to moderately depressed subjects (53.8%), 60.32% were hindus, 37.22% were muslims followed by 2.44% Christians. More than 74% of subjects lived in a nuclear family and 87.22% were perusing graduation. Monthly per capita family income of 18.3% population was below 20,000 of which 29.6% reported to be mild to moderately depressed. Approximately 43% people stated monthly per capita family income above 60,000. The dietary profile of the subjects enrolled in the present study indicated lower energy and macronutrients consumption among students who reported severe and mild to moderate depression in comparison to normal. The mean energy intake of the subjects falling in mild to moderate and severe depressed category reported to be 9.21% and 19.5% less than the prescribed RDA respectively. When compared with RDA for age 19-30years, lower intake of CHO was reported by 21.6% and 26.33 % amongst the mild to moderate and severe depressed subjects. Difference in intake of protein from RDA was 15.89 % and 29.09% for mild to moderately and severe depressed category respectively which is much lower than RDA.

Fat intake was observed to be 70.58%, 67.7% and 17.7% higher than RDA in normal, mild to moderate and severely depressed subjects respectively. The data from the current result also revealed less consumption of micronutrients by subjects experiencing severe depression in comparison to normal. With reference to RDA, decrease in consumption of Calcium was seen by 2 % and 5.83%, iron by 61.9% and 63.28%, magnesium by 4.51% and 21.29%, and zinc by 54.3% and 55.3 % respectively for mild-moderate and severely depressed enrolled subjects. Mean intake of omega 3 and omega 6 was also quite less in mild to moderate and severely depressed subjects. Among the screened subjects, 43.5% people in severe depression category were vegetarian, followed by 37.5% and 18.75% ovo lacto vegetarian and non-vegetarian respectively. In mild to moderate depression category 76.3% subjects said they were vegetarian followed by 16.57% ovo lacto vegetarian and 7% non-vegetarian.

Regarding the consumption of tea/coffee, 14.4% subjects falling in the category of mild to moderate depression, reported they have 1 cup daily, while 28.26% have more than 3 cups. Under severe depression category 15.62% subjects said they consume 1 cup/day, 25% people had more than 3 cups. As reported by people with mild to moderate depression aerated beverages were consumed frequently by 14.94% and 80.43% had them on moderate base. Only 14% subjects reported practicing yoga or breathing exercise daily while 56.79% said they were never involved in it. Participants (22.55%) walk or jog daily, 54.6% moderately and 22.82% never had this habit.

Result of the present study also showed direct association between depression and

intestinal motility, severe constipation (18.75%) was reported by subjects falling in severe depression category, whereas 12.5 % of subjects falling in mild- moderate depression zone and only 2% people among normal population reported it. Subjects 73.49%, 48.64%, and 9.37% reported normal stool frequency in normal, mild to moderately depressed and severely depressed category respectively. Subjects who reported frequent consumption of pre/probiotics and medical history of non-communicable diseases were not included for any of the intervention phases. Significant negative correlation of depression scores was seen with fecal log count of *Lactobacillus* (p value<0.000). Positive significant correlation was observed with log count of E. coli and defecation profile (p value<0.000).

No significant Pearson Correlation of cortisol was observed with any of the parameter. Fecal log count of gut friendly bacteria *Lactobacillus* demonstrated negative correlation with depression, *E. coli* and defecation profile (p value<0.000). *Bifidobacterium* count were positively related with *Lactobacillus* and negatively with depression and *E. coli.*

PHASE -III: Impact evaluation of intervention trials with ambil (prebiotic enriched fermented beverage) on the fecal microbial counts (*Lactobacillus, Bifidobacteria and Enteric pathogens*), depression status, serum cortisol and defecation profile of mild to moderately depressed subjects.

Mild to moderately depressed subjects who gave informed written consent for participation and followed inclusion and exclusion criteria, were enrolled in the study. Systemized random sampling technique was used to divided participants into experimental group 1, experimental group 2, experimental group 3, experimental group 4 and control group.

In Phase III of the study subjects falling in experimental group 1 were intervened with 200 ml prebiotic enriched fermented beverage (ambil) for the duration of 45 days. Subjects showed a significant decrease in mean depression scores and cortisol values by 46.45% and 6.56 % respectively. The fecal log count of *Lactobacillus* and *Bifidobacterium* showed a significant increase of 10.05% and 36.15% respectively and a reduction of 2.88% in *E. coli*. Improvement in stool frequency was seen by 0.28%. When compared with the control group high improvement in depression score and log count of *Bifidobacterium* was seen (p value <0.001) post intervention in experimental group. E. coli also reduced pvalue<0.01. However, no significant difference was seen in the levels of serum cortisol post intervention within experimental group as well as in comparison with control group**.**

PHASE IV: Studying the prebiotic efficacy of FOS supplementation by determining fecal microbial counts (*Lactobacillus, Bifidobacteria* and *E. coli*), depression status, serum cortisol and defecation profile of mild to moderately depressed subjects before and after supplementation.

Experimental group 2 was exposed to 10 ml liquid fructooligosaccharide supplementation for 45 days. No significant difference was observed in any of the parameters assessed pre intervention between the experimental group 2 and control group. Though, significant decrease in percentage of depression scores and log count of *E. coli* was detected by 18.69% and 3.72 % respectively post intervention with FOS. Experiential increase in colonization of *Lactobacillus and Bifidobacterium* was seen by 6.8% and 6.13% which was highly significant $p \, value < 0.001$.

PHASE V: Pre and post assessment of gut microbiome log count with respect to *Lactobacillus, Bifidobacteria and E. coli*, state of depression, blood serum cortisol and degree of constipation in mild to moderately depressed subjects after intervention with tetra packed buttermilk.

Buttermilk supplementation, bought significant diminution $(p \, value < 0.001)$ in the depression scores and log count of *E. coli* by 13.43 % and 1.58% respectively, decrease in the levels of serum cortisol was also marked by 7.38% although it was not significant. Gut health improved with significant increase in colonization with *Lactobacillus* and *Bifidobacterium* by 5.47% and 5.68%. No significant changes were seen on comparison with the control group with respect to any of the parameters pre and post intervention.

Phase VI: Assessing the potential of fresh buttermilk in modulating the gut floras (*Lactobacillus, Bifidobacteria and E. coli*), checking depression, blood serum cortisol and defecation profile in mild to moderately depressed subjects.

Fresh buttermilk intervention for 45 days marked reduction in the depression scores and log count of *E. coli* by 14.21 % and 2.71% respectively, decrease in the levels of serum cortisol was also seen by 4.49% although it was not significant. Gut health improved with significant increase in colonization with *Lactobacillus* by 5.57% and *Bifidobacterium* by 4.51%.

PHASE VII: Evaluating which intervention had highest impact in reversing depression scores, cortisol levels and modulating gut microbiota composition.

Post hoc test was used to determine the differences in mean scores of depressions, serum cortisol levels and gut microbiota post supplementation with ambil, FOS, fresh buttermilk, and tetra packed buttermilk. The analysis was made to figure out which supplementation proved out to be effective in reversing depression scores, cortisol levels and setting the gut microbial modulation to a positive balance.

Ambil was most effective in lowering depression followed by tetra packed buttermilk, FOS and fresh buttermilk. FOS proved out to be most effective intervention in reducing cortisol levels tailed by tetra packed buttermilk, ambil and fresh buttermilk. the therapeutic role of Ambil increased the colonization of good gut bacteria *Bifidobacterium*. Log count of *Lactic acid bacteria* increased highest in the experimental group supplemented with fresh buttermilk followed up by tetra packed buttermilk, FOS and Ambil supplementation groups. Highest reduction in the count of pathogenic bacteria *E. coli* was reported in experimental group on FOS supplementation followed by Ambil, fresh buttermilk and tetra packed buttermilk supplementation.

PHASE VIII: Developing an audio-visual animated aid as a means of IEC material to make general people aware about increasing trend of depression and how to cope up with it.

Animated movie was developed using the software *Animaker*.

Discussion

Mental health, in particular depression, is a matter of concern worldwide and still the most overlooked wellbeing aspect. One in every seven adolescent meets the diagnostic criteria for a mental health disorder (Polanczyk et al., 2015) and one in every twenty is suffering from depression in India (NMHS, 2016).

Early detection and prompt access to professional treatment can eliminate mental health from getting worse. Various institutional-based studies point towards an increasing prevalence of depression among youth. Major concerns are negligence, social stigma and the fear of seeking professional help. A recent 2020 study identified 53 eligible studies which highlights the complex array of reluctant nature of youth for seeking help for mental health difficulties. Almost all studies (96%) reported barriers related to individual factors, such as limited mental health knowledge and broader perceptions of help-seeking. Studies (92%) pointed towards perceived social stigma and embarrassment while talking about mental health. Young people's impressions of therapeutic interaction with experts, such as perceived secrecy and the ability to trust an unfamiliar person, were captured in (68%). The fourth issue (58%) dealt with systemic and structural barriers and facilitators, such as the financial expenses of mental health care, logistical challenges, and the availability of professional assistance (Radez et al., 2020).

Given the current state of youth's relationship with their mental health, the current study was designed to gain insight into the intriguingly increased frequency of depression among the students at the Faculty of Family and Community Sciences, The MS University of Baroda. The depression assessment was carried out on 683 students through self-assessed Beck's Depression Inventory in the present set of research. According to the assessment, a large number of university students (53.85%) scored between 11 and 30 on the basis of which they were classified as mild to moderately depressed. Nearly five percent were suffering from severe depression, and they were unaware of it. Only 283 (41.43%) of the total screened subjects scored normal, indicating the absence of depression.

These results are almost comparable to the data of other institutional based studies. Bhopal University conducted a research using a theoretical Depressive Experiences Questionnaire that revealed 31% of students were depressed at the age of 21 (Alghamdi, N. G. 2015). Punjab University, Chandigarh assessed the mental health of 400 students from nine departments. The overall prevalence of depression, anxiety and stress came out to be 59.2%, 86.5% and 52.7% respectively (Singh, M et al., 2017). A cross-sectional survey conducted

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amongst 331 undergraduate students at college in Gujarat using the Patient Health Questionnaire reported the overall prevalence of depression to be 64% with the highest level of depression among first year students whereas, moderate to severe depression was found in 26.6% of students (Vankar et al., 2014). The rising trend in youth depression requires attention to both underlying issues and effective solutions to tackle the condition before it has severe societal impact.

The present chapter also dialogues the various aspects attached to youth depression. The majority of the research participants were in the age group of 19-25. Screening revealed that among 60.32% of the mild to moderately depressed subjects (53.85%) were hindus, 37.22% were muslims, and 2.44% were christians. Significant differences in family structure were demonstrated in our study. In the mild to moderate depression category, approximately 35% came from a joint family, nearly 16% from an extended joint family, and 74.37% came from a nuclear family. In the normal mental condition category, nearly 65% came from a joint family, more than 80% from an extended joint family, and 17.82% came from a nuclear family set up. This study's subjects (87.22%) were pursuing graduation. Monthly per capita family income was less than 20,000 for 18.3% of the population, with 29.6% reporting mild to moderate depression. In line with the data, lies the results of research carried out by Kaur s et al 2014., which correlated depression in 200 students of various educational institutes in Amritsar, Punjab using Patient Health Questionnaire. The majority of students were in the age group 18-24. The overall prevalence of depression noted was 16.5%. Out of all studied subjects, 81 (40.5%) were male and 119 (59.5%) were female. All of the subjects were unmarried (100%), the majority of whom lived in cities (77.5%), came from nuclear families (79.5%), practiced hinduism (57%), and belonged to a middle socioeconomic class (67%).

As revealed in a fantastic research study, meditation and breathing exercises practice done over 30 minutes a day for eight weeks in a row has been shown to modulate the density of the brain's grey region associated with learning, memory, and cognition. The hippocampus, an area of the brain associated with learning which tends to shrivel in size with age, also expands with meditation (Anna Lardone et al., 2018). In the present set of research, nearly thirty percent of participants who reported no depression were practicing breathing exercise daily, which ranged to 14% and 12% in mild to moderately depressed and severely depressed subjects respectively. Studies have also examined the efficacy of walking 20 to 40 minutes 3 times per week for 6 weeks in alleviating somatic symptoms of depression on the Beck Depression Inventory (McNeil JK 1991). According to the current study, 22.55% of mild to

moderately depressed participants walk or jog daily, 54.6% moderately, and 22.82% never did. Among severely depressed subjects 71% never walk or jog as part of their daily activity.

This research also opens an argument why nutritional allowance should not be taken for granted when it comes to proper brain functioning. Data received highlights the role of both macro and micro nutrient consumption on one's mental status. The dietary profile of subjects enrolled indicated that RDA was not met in severe and mild to moderately depressed individuals when compared to normal. Carbohydrate rich foods trigger the secretion of brain chemicals serotonin and tryptophan. Therefore, a diet low in carbs interferes with the proper functioning of brain chemical messengers (T. S. Sathyanarayana Rao et al., 2008). Our results provide evidence of this fact, 21.6% mild to moderate and 26.33% severely depressed subjects reported lower intake of CHO. Protein is linked to neurotransmitter synthesis, a lack of amino acids in the diet has an effect on mood. A positive association exists between low protein intakes and the prevalence of mental illnesses (Nanri A 2015). The difference in protein consumption of depressed individuals was much lower than RDA in the current study, in mild to moderately depressed by 15.89% and in the severe depressed category by 29.09%. Similar findings were reported by D'Anci KE et al in 2009 where low carbohydrates were associated with greater risk of mood disorders including anxiety and depression. Empirical evidence most strongly supports a positive association between micronutrient deficiency with mental health. Findings support the importance of adequate consumption of zinc, selenium and magnesium in the promotion of mental health (Wang, J et al., 2018). Looking at the data from the current result, consumption of micronutrients was also reportedly less in depressed individuals than normal. The decrease in consumption of calcium was seen by 2% and 5.83%, iron by 61.9% and 63.28%, magnesium by 4.51% and 21.29%, and zinc by 54.3% and 55.3% respectively for mild-moderate and severely depressed enrolled subjects. The mean intake of omega 3 and omega 6 was also quite less in mild to moderate and severely depressed subjects.

Studies have investigated patients with depression have indication of abnormal intestinal transit. Data from the National Health and Nutrition Examination Survey (NHANES), a representative sample of the US population, studied the relationship between depression and bowel habits among 495 individuals. Investigators found that more individuals with depression reported disordered bowel habits compared with individuals without depression (24.6% vs. 12.6%). The result of the present study also showed direct association between depression and intestinal motility, severe constipation was reported by subjects falling in severe depression category (18.75%), whereas 12.5 % of subjects falling in mild- moderate depression zone and only 2% people among normal population reported constipation.

Gut microbial modulation is the key to the establishment of better brain functionalities. Getting the right gut bacteria is one of the favorable options and the core connection for gut brain axis. When the daily diet of subjects who counted mild to moderate scores on Beck's Depression Inventory was looked upon in terms of consumption frequency of prebiotic, and fermented milk-based beverages which forms the source of probiotic; like curd, buttermilk, yogurt, shrikhand, matho and lassi it was clear subjects who frequently consumed such foods were less likely to fall under depression category as compared to who consumed it less frequently. Other researches also support this algorithm *Kato-Kataoka A et al., 2016* reported that consumption of fermented milk prevents the onset of physical symptoms in medical students under academic examination stress. Issue published in *Psychiatry Research,* August 2015 showed that college undergraduates who ate the most fermented foods, including sauerkraut, yogurt and pickles, were less likely to suffer from social anxiety, fear or depression.

To elicite the importance of establishing gut flora to a positive balance and further asses their role in combating depression other phases of the study were planed and implemented which focused on looking up the beneficial effect of intervention with psychobiotics -FOS, buttermilk, fermented beverage and combinations to mild to moderately depressed subjects. The biochemical parameter involved was blood serum cortisol as it is an indicator for stress. Defecation profile with respect to constipation, fecal log count of good gut floras *Lactobacillus* and *Biffidobacteria* and enteric pathogen counts of E. coli was studied before and after supplementation intervals.

The process of fermentation in ambil (a cereal buttermilk based beverage enriched with FOS) amplifies the growth of Bifidobacterium strains: *Bifidobacterium stercoris, Bifidobacterium faecale, Bifidobacterium adolescentis, Bifidobacterium ruminantium, Bifidobacterium catenulatum, Bifidobacterium kashiwanohense, Bifidobacterium pseudocatenulatum, Bifidobacterium angulatum and* Lactobacillus strains*: Lactobacillus acidophilus, Lactobacillus acidophilus, Lactobacillus acidophilus, Lactobacillus acidophilus, Lactobacillus kitasatonis, Lactobacillus gallinarum strain, Lactobacillus gallinarum strain, Lactobacillus crispatus strain, Lactobacillus crispatus* strain (Hirdyani and Sheth thesis, 2018). The presence of both prebiotic and probiotics has made it symbiotic. Moreover, fermentation enhances the microflora in milk which act both as preservators and transformers as they have the power to amplify the bioavailability and activity of various biogenic metabolites which have therapeutic potential, the value of which is also associated with mental health *(Selhub E. M. et al., 2014)*. Microbial fermentation is one of the most promising methods for producing nutritional peptidomes (Panchaud et al., 2012). The *Lactic acid*

bacteria proteolytic system acts on casein present in milk and milk derivatives to supply bioactive peptides (Clare and Swaisgood; 2000). These peptides have proved their worth in gut-brain communication, enhancing brain cognitive function (Dinan, and Cryan; 2017). In a randomized, double-blind, placebo-controlled study, the consumption of fermented milk with multiple probiotic strains and prebiotic fibers has been shown to improve constipation in Parkinson's patients (Barichella, M et al., 2016). The result of the present study has also quantified the presence of bioactive peptides casoxin C (21.3mg/kg) and ß casomorphin (6.2mg/kg) in ambil. According to research, beta-casomorphins (BCM) are the most well-studied myorelaxant peptides (Pessione and Cirrincione 2016). Casoxins can be employed to counteract depression (Chiba and Yoshikawa, 1986). BCM7 has been detected and quantified in a number of pasteurized milk products. However, to date, specific enzymes contributing to the formation of BCM7 in dairy products are still unknown (Nguyen et al., 2015). Nguyen et al in 2014 identified β-casomorphin in yogurt. Systemic administration of low dose of bovine β-casomorphin-5 has been shown to improve the disturbance of learning and memory in mice (Sakaguchi et al., 2006). Products containing β-casomorphin-7 also have the potential to improve intestinal health by preventing the adherence of pathogens to the intestinal surface and thus eliminating the onset of intestinal infections. Oral administration of this peptide at the rate of 0.1–100 μmol/L to rat pups enhanced the number of goblet cells and Paneth cells along the small intestine (Plaisancie et al., 2013).

Despite the loss of probiotics due to the tetra packing of the ambil supplemented, we can still claim the product's goodness for health because released biogenic metabolites make the product directly beneficial without the need for live bacteria (Korhonen and Pihlanto, 2003). These molecules constitute a promising frontier for treating both stress-related behaviours such as anxiety and depression and season-related mood disorders (Pfluger et al. 2012). In general, these peptides are able to control the gut–brain axis at several levels, including gut–brain communication, brain cognitive function, and behavior (Cryan and Dinan, 2012). It has been demonstrated that the biological effect is mediated by activation of serotonin and $GABA_A$ receptors, causing release of endogenous serotonin, dopamine, and GABA (Mizushige et al., 2013). Present research has shown that consumption of ambil having bioactive peptides casoxin C and ß casomorphin helps in the reduction of cortisol and depression scores. The supplementation of 200 ml of this beverage to 30 subjects suffering from mild to moderate depression for 45 days resulted in a significant decrease in mean depression scores and cortisol values of 46.45% and 6.56%, respectively. The fecal log count

of *Lactobacillus* and *Bifidobacterium* showed a significant increase by 10.05% and 36.15% respectively and a reduction of 2.88% in E. coli was seen.

Similar studies favour our results. In a double-blind study involving over 40 healthy male subjects, two groups were subjected to psychological and physical stress tests, with cortisol concentrations, heart rate, and blood pressure levels measured at specific intervals. Each of these three stress response markers were substantially lowered in the group taking the proprietary bioactive milk peptide compared to controls. In comparison, the milk peptide group experienced a net reduction in cortisol of 20.69%. In other words, the release of cortisol was kept under control by the *milk peptides,* limiting its detrimental effects (Messaoudi M et al., 2005). Kim JH et al in 2007 also showed that bioactive peptides have a role to play in mental health. A group of 60 women suffering from a constellation of stress-related problems were given 150 mg per day αs1-casein hydrolysate bioactive milk peptide compound. Compared to controls, after 30 days, significant improvements were observed in cognitive function (62.5% improvement—16.3% over placebo).

So, the presence of the bioactive peptides in ambil may be one probable reason for improved mental status. Intake of ambil has shown direct relation with improved defecation profile by releasing constipation. A significant improvement of 0.287% was noted. In view of our results, another study on 274 adults with IBS found that consuming 4.4 ounces of yogurt-like fermented milk daily for 6 weeks improved IBS symptoms, including bloating and stool frequency (Guyonnet et al., 2007).

It is well established now that the intestinal microbial community formulates the basis of crosstalk between the gut and brain. The idea that certain nutrients can augment the gut microflora existed before the definitions for such nutrients were proposed. Prebiotics, which can be described as the feed for the intestinal microbiota, hold the potential of increasing saccharolytic activity within the gut by acting as a catalyst in modulating gut microflora to a positive balance.

Wang and Gibson in 1993highlighted the potential of oligofructose and inulin in promoting the growth of the health promoting genus *Bifidobacterium infantis* while maintaining low populations of potential pathogens *Escherichia coli* and *Clostridium* difficile. A 1995 study on human volunteers found that those who were fed strictly controlled 15g chicory fructooligosaccharide supplements per day for 15 days had a significant increase in the number of *Bifidobacterium*. Only 4g of fructooligosaccharide per day is sufficient to observe an increase in the gut bifidobacterial as pointed out by a Japanese study (Tuohy KM et al., 2003).

In the present research on FOS supplementation (10 ml liquid) for 45 days, an increase in colonisation of *Lactobacillus* and *Bifidobacterium* was noted by 6.8% and 6.13%, respectively, while a reduction in E. coli was seen by 3.13%, which was highly significant (p value 0.001). Supportive research has also claimed prebiotic supplementation as an effective method of re- introducing healthy bacteria back into the gut microbiome as shown by Sheth et al., 2014. Supplementing 12 gm FOS to 65 obese adults for a period of 12 weeks resulted in a significant increase in *Lactic acid bacteria* and *Bifidobacterium* by 14% and 10% respectively. With a 20% reduction in enteric pathogens. Data is supported by a recent investigation carried on 80 participants by Tandon et al., 2019 FOS increased the relative abundance of *Bifidobacterium* and *Lactobacillus* while its withdrawal led to its reduction.

Hype surrounding the human microbiome influencing mental health stems from the belief that gut microbiota can modulate host brain function and behavior via the microbiota-gut-brain axis. There is a growing body of evidence documenting the ability of prebiotics to normalize the dysbiosis microbiota associated with psychological disorders such as anxiety, depression, stress, autism, learning, and memory (Liu X et al. 2105). The first report of the psychobiotic properties of prebiotics was examined on Sprague–Dawley rats. Prebiotic ingestion increased hippocampal BDNF expression and BDNF mRNA expression, which play an essential role in maintaining synaptic plasticity and optimal memory function (Li and Tsien.,2009). A supporting study found that rats fed the prebiotic during lactation had significantly improved maze-learning and object-recognition even one year later, indicating that prebiotic ingestion has very long-lasting benefits. The result of the present study also demonstrated a significant decrease in mean depression scores and cortisol values by 18.69% and 1.76% respectively with the positive shift in microbial diversity on fructooligosaccharide supplementation. Consonant with our research results, there was a significant increase in mean log counts of beneficial gut microbiota with improvement in SAGE scores of the mild to moderate Alzheimer's subjects who were on FOS supplementation. Another prebiotic, galactooligosaccharide also lowered cortisol awakening reactivity and attenuated vigilance, which is a behavioural marker of anxiety and depression (Schmidt K et al. 2015). Chen D in 2017 through his work confirmed the secretion of neurotransmitter exerting effective memory improvements on consumption of Fructooligosaccharide. The positive impact of FOS-mediated increased bacterial diversity reinforces the role of prebiotics in conferring a positive effect on mental health.

Buttermilk is usually processed by natural fermentation with lactic acid bacteria. The importance of this age-old Indian dairy beverage in defining gut bacteria cannot be overstated;

studies have demonstrated its ability to prevent gastrointestinal infections by inhibiting bad bacteria such as Helicobacter pylori (Jenks, P. J. 2002).The bacterial strains isolated from buttermilk used for supplementing the subjects of current research as identified by Hirdyani and Sheth,2018 were *Bifidobacterium pseudocatenulatum, Bifidobacterium catenulatum, Bifidobacterium angulatum, Bifidobacterium kashiwanohense, Bifidobacterium ruminantium, Bifidobacterium merycicum, Bifidobacterium callitrichos, Bifidobacterium stercoris, Lactobacillus acidophilus, Lactobacillus acidophilus, Lactobacillus gallinarum, Lactobacillus gasseri, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus paracasei, Lactobacillus crispatus, and Lactobacillus crispatus.* In our research, the role of buttermilk consumption was assessed on gut profile and depression. Buttermilk supplementation reduced the log count of pathogenic bacteria *E.coli* by 2.71%, and gut health improved with a significant increase in colonization of the gut with *Lactobacillus* and *Bifidobacterium* by 5.57% and 4.51% respectively. Subjects who consumed tetra packed buttermilk also showed an increase in log count of *Lactobacillus* and *Bifidobacterium* by 5.4% and 5.68% respectively and a reduction of *E. coli* by 1.58% was confirmed. The reduction in depression status and cortisol scores for subjects who consumed fresh buttermilk were 14.21% and 4.49% in comparison to subjects supplemented with tetra packed buttermilk which was 13.43% and 7.38%.

The positive role of dairy based fermented foods on mental health was also confirmed through similar studies. Consumption of 200 ml/day of fermented milk for 12 weeks showed significant improvement in the Mini-Mental State Exam from 8.7 to 10.6 (Akbari E et al. 2016). Yogurt consumption for two weeks led to greater increases in serotonin levels of healthy individuals (Kim, H. et al., 2018).

Pearson's correlation was taken into account to compare the relativity of depression with various parameters. As expected, the fecal log count of *Lactobacillus* showed a negative correlation with depression scores, while depression was inversely proportional to the log count of *E. coli* and constipation severity. Though a difference in cortisol levels was observed in all the intervention therapies, this difference was non-significant with any of the supplements given as well as when compared with the control group. Our findings contradicted with the study results conducted by Kristin Schmidt et al in 2014, which found that introducing prebiotic galactooligosaccharide reduced the salivary cortisol awakening response when compared to a placebo. Research performed in 2019 by Asma Kazemi on prebiotics and probiotic consumption showed a decrease in levels of cortisol by 20% and 19% in experimental groups respectively.

With an increase in the depression rate among young adults, there is a strong need to bridge the gap between communication and the lack of avenues for treatment. People still feel it a taboo to talk about mental health. Educating the community about depression is the need of the hour. The major concern is that people don't even realize that they are diseased. We made an attempt to create awareness about depression in the general population through the last phase of our research. An audio-visual animated movie was created in the hopes that the information- education -communication (IEC) developed as part of our study will be widely distributed among the general public, allowing people to develop a common understanding of depression, feel motivated to talk about it, and take precautionary measures ahead of time. Multimedia based communication has got better retention possibility compared to other methods. An animated movie developed using the software *Animaker* tried to cover every aspect of this disease, including symptoms, the need to communicate with subject expert, the role of nutrition and exercise and how to formulate new habits for betterment of self. The movie is named 'RESTART', which aims to ring the wake-up call to restart with new hope, new strength and new thoughts to overcome depression.

Conclusion and Recommendations

Rising rates of depression among youth have become a concerning issue as it imposes a negative effect not only on physiological and psychological wellbeing but also provokes one to threaten own life. In the present study, screening of the subjects aged 19-30 for depression using Beck's Depression Inventory showcased that more than 50 % population is affected by mild to moderate depression. There is a need for early screening and conventional therapy to improve the mental status of this vulnerable population.

This research has elicited the importance of the gut microbiome in improving depression status. Proving the signaling between gut and brain. Maintaining the right gut bacteria is the key to good mental health. Fermentation magnifies the benefit of dairy promoting the bioavailability of biogenic metabolites (BM) which are further associated with mental health. This research also focused on the quantification of such BM in ambil (prebiotic enriched rice-based dairy fermented beverage)

Prebiotics and fermented beverages have proven out to be an effective remedy in modulating the gut environment to a positive balance thereby, reducing depression. Mild to moderately depressed subjects who consented to participate in the research were randomly divided into four different intervention groups. They were given ambil, FOS, buttermilk fresh, and tetra packed for a period of 45 days. A marked increase in the colonization of beneficial microorganisms was seen along with reduced depression status in all groups, with the highest improvement in the group supplemented with ambil. Cortisol a stress biomarker was also assessed, however, no significant correlation was seen with it. An animated movie with the hope of making people aware of this common mental illness was also developed.

However, further studies shall be undertaken to demonstrate the clinical efficacy of fermented dairy beverages and prebiotics with respect to mental health. More reliable bio-parameters could be used to seal the proof of axis between the gut and brain.

References

Akbari, E., Asemi, Z., Kakhaki, R. D., Bahmani, F., Kouchaki, E., Tamtaji, O. R., Hamidi, G. A., & Salami, M. (2016). Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: A randomized, double-blind and controlled trial. *Frontiers in Aging Neuroscience*, *8*(NOV). https://doi.org/10.3389/fnagi.2016.00256

Barichella, M., Pacchetti, C., Bolliri, C., Cassani, E., Iorio, L., Pusani, C., Pinelli, G., Privitera, G., Cesari, I., Faierman, S. A., Caccialanza, R., Pezzoli, G., & Cereda, E. (2016). Probiotics and prebiotic fiber for constipation associated with Parkinson disease. *Neurology*, *87*(12), 1274–1280. https://doi.org/10.1212/WNL.0000000000003127

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology*, *28*(2), 203–209. www.annalsgastro.gr

Chen, D., Yang, X., Yang, J., Lai, G., Yong, T., Tang, X., Shuai, O., Zhou, G., Xie, Y., & Wu, Q. (2017). Prebiotic effect of Fructooligosaccharides from Morinda officinalis on Alzheimer's disease in rodent models by targeting the microbiota-gut-brain axis. *Frontiers in Aging Neuroscience*, *9*(DEC), 403. https://doi.org/10.3389/fnagi.2017.00403

ChibaH.,andYoshikawaM.(1986)."Biologicallyfunctionalpeptidesfromfood proteins: newopioidpeptidesfrommilkproteins,"in ProteinTailoringforFood and MedicalUses, edsR.E.FeeneyandJ.R.Whitaker(NewYork:Marcel Dekker Inc.),123–153.

Clapp, M., Aurora, N., Herrera, L., Bhatia, M., Wilen, E., & Wakefield, S. (2017). Gut Microbiota's Effect on Mental Health: The Gut-Brain Axis. *Clinics and Practice*, *7*(4), 131–136. https://doi.org/10.4081/cp.2017.987

Clare D. A., Catignani G. L., Swaisgood H. E. Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Curr. Pharm. Des.* 2003;9:1239–1255.

Clare D. A., Swaisgood H. E. (2000). Bioactive milk peptides: a prospectus. *J. Dairy Sci.* 83 1187–1195. 10.3168/jds.S0022-0302(00)74983-6

De Palma, G., Collins, S. M., Bercik, P., & Verdu, E. F. (2014). The microbiota-gut-brain axis in gastrointestinal disorders: Stressed bugs, stressed brain or both? *Journal of Physiology*, *592*(14), 2989–2997. https://doi.org/10.1113/jphysiol.2014.273995

Dehghan, P., Pourghassem Gargari, B., & Asghari Jafar-abadi, M. (2014). Oligofructose-enriched inulin improves some inflammatory markers and metabolic endotoxemia in women with type 2 diabetes mellitus: A randomized controlled clinical trial. *Nutrition*, *30*(4), 418–423. https://doi.org/10.1016/j.nut.2013.09.005

Dinan, T. G., and Cryan, J. F. (2017). Gut instintcs: microbiota as a key regulator of brain development, ageing and neurodegeneration. *J. physiol.* 595, 489–503. doi: 10.1113/JP273106

Forsythe, P., Bienenstock, J., & Kunze, W. A. (2014). Vagal pathways for microbiome-brain-gut axis communication. *Advances in Experimental Medicine and Biology*, *817*, 115–133. https://doi.org/10.1007/978-1-4939-0897-4_5

Gallagher RP. *National Survey of Counseling Center Directors: American College Counseling Association.* The International Association of Counseling Services, Inc.; 2011.

Guyonnet, D., Chassany, O., Ducrotte, P., Picard, C., Mouret, M., Mercier, C. H., & Matuchansky, C. (2007). Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: A multicentre, randomized, double-blind, controlled trial. *Alimentary Pharmacology and Therapeutics*, *26*(3), 475–486. https://doi.org/10.1111/j.1365-2036.2007.03362.x

Harsha, H. (2018). Probiotic profiling and organoleptic evaluation of traditional cereal based fermented drink and its market potential. *University*. http://hdl.handle.net/10603/259698

Hogan, M. F. (2003). The President's New Freedom Commission: Recommendations to Transform Mental Health Care in America. In *Psychiatric Services* (Vol. 54, Issue 11, pp. 1467–1474). American Psychiatric Publishing. https://doi.org/10.1176/appi.ps.54.11.1467

James, T. (2020). A Study to Assess the Prevalence of Depression and Anxiety among Medical Students of a Teaching Hospital in South India. *Original Research Article J. Evid. Based Med. Healthc*, *7*. https://doi.org/10.18410/jebmh/2020/304

Jenks, P. J. (2002). Causes of failure of eradication of Helicobacter pylori. In *British Medical Journal* (Vol. 325, Issue 7354, pp. 3–4). BMJ Publishing Group. https://doi.org/10.1136/bmj.325.7354.3

Kato-Kataoka, A., Nishida, K., Takada, M., Suda, K., Kawai, M., Shimizu, K., Kushiro, A., Hoshi, R., Watanabe, O., Igarashi, T., Miyazaki, K., Kuwano, Y., & Rokutan, K. (2016). Fermented milk containing Lactobacillus casei strain Shirota prevents the onset of physical symptoms in medical students under academic examination stress. *Beneficial Microbes*, *7*(2), 153–156. https://doi.org/10.3920/BM2015.0100

Kaur.S, Deepti S ,S Mohan Lal (2014). *Prevalence and Correlates of Depression among College going students of District Amritsar, India*. (n.d.). Retrieved June 17, 2021, from https://www.researchgate.net/publication/270052404_Prevalence_and_Correlates_of_Depres sion_among_College_going_students_of_District_Amritsar_India

Kazemi, A., Noorbala, A. A., Azam, K., Eskandari, M. H., & Djafarian, K. (2019). Effect of probiotic and prebiotic vs placebo on psychological outcomes in patients with major depressive disorder: A randomized clinical trial. *Clinical Nutrition*, *38*(2), 522–528. https://doi.org/10.1016/j.clnu.2018.04.010

Kelly, J. R., Kennedy, P. J., Cryan, J. F., Dinan, T. G., Clarke, G., & Hyland, N. P. (2015). Breaking down the barriers: The gut microbiome, intestinal permeability and stress-related psychiatric disorders. In *Frontiers in Cellular Neuroscience* (Vol. 9, Issue OCT, p. 392). Frontiers Media S.A. https://doi.org/10.3389/fncel.2015.00392

Kim, H. K., Kim, S. H., Jang, C. S., Kim, S. I., Kweon, C. O., Kim, B. W., & Ryu, J. K. (2018). The combined effects of yogurt and exercise in healthy adults: Implications for biomarkers of depression and cardiovascular diseases. *Food Science and Nutrition*, *6*(7), 1968–1974. https://doi.org/10.1002/fsn3.772

Korhonen H. Pihlanto A (2003). Food-derived bioactive peptides - Opportunities for designing future foods. *Current Pharmaceutical Design*, : 1297–1308.

Lardone, A., Liparoti, M., Sorrentino, P., Rucco, R., Jacini, F., Polverino, A., Minino, R., Pesoli, M., Baselice, F., Sorriso, A., Ferraioli, G., Sorrentino, G., & Mandolesi, L. (2018). Mindfulness meditation is related to long-lasting changes in hippocampal functional topology

during resting state: A magnetoencephalography study. *Neural Plasticity*, *2018*. https://doi.org/10.1155/2018/5340717

Liu X, Cao S, Zhang X. Modulation of gut microbiota–brain axis by probiotics, prebiotics, and diet. *J Agr Food Chem* 2015;63:7885-95.

McLoughlin, R. F., Berthon, B. S., Jensen, M. E., Baines, K. J., & Wood, L. G. (2017). Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: A systematic review and meta-analysis. In *American Journal of Clinical Nutrition* (Vol. 106, Issue 3, pp. 930–945). American Society for Nutrition. https://doi.org/10.3945/ajcn.117.156265

McNeil, J. K., LeBlanc, E. M., & Joyner, M. (1991). The effect of exercise on depressive symptoms in the moderately depressed elderly. *Psychology and Aging*, *6*(3), 487–488. https://doi.org/10.1037//0882-7974.6.3.487

Mini Sheth* , Abnita Thakuria1 , Vasundhra Chand2 and Dr. Mridu Paban Nath3 (2015). fructooligosaccharide (fos)- a smart strategy to modulate inflammatory marker and lipid profile in non insulin dependent diabetes mellitus (niddm) subjects residing in assam, india- a randomized control trial World Journal of Pharmaceutical Research. (Vol. 4, Issue 5).

Mizushige, T., Sawashi, Y., Yamada, A., Kanamoto, R., & Ohinata, K. (2013). Characterization of Tyr-Leu-Gly, a novel anxiolytic-like peptide released from bovine αS-casein. *FASEB Journal*, *27*(7), 2911–2917. https://doi.org/10.1096/fj.12-225474

Nanri A, Eguchi M, Kuwahara K, Kochi T, Kurotani K, Ito R, et al. Macronutrient intake and depressive symptoms among Japanese male workers: the Furukawa Nutrition and Health Study. Psychiatry Res. 2014;220(1–2):263–8

Ooi, L. G., & Liong, M. T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: A review of in Vivo and in Vitro Findings. In *International Journal of Molecular Sciences* (Vol. 11, Issue 6, pp. 2499–2522). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ijms11062499

Panchaud, A., Affolter, M., & Kussmann, M. (2012). Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects. In *Journal of Proteomics* (Vol. 75, Issue 12, pp. 3546–3559). https://doi.org/10.1016/j.jprot.2011.12.022

Pessione, E., & Cirrincione, S. (2016). Bioactive molecules released in food by lactic acid bacteria: Encrypted peptides and biogenic amines. In *Frontiers in Microbiology* (Vol. 7, Issue JUN). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2016.00876

Pessione, E., & Cirrincione, S. (2016). Bioactive molecules released in food by lactic acid bacteria: Encrypted peptides and biogenic amines. In *Frontiers in Microbiology* (Vol. 7, Issue JUN). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2016.00876

Pfluger P. T., Schriever S. C., Tschöp M. H. (2012). Nutropioids, hedonism in the gut? *Cell Metab.* 16 137–139. 10.1016/j.cmet.2012.07.011

Polanczyk GV, Salum GA, Sugaya LS et al (2015) Annual research review: a meta-analysis of the worldwide prevalence of mental disorders in children and adolescents. J Child Psychol Psychiatry Allied Discip 56:345–365. https://doi.org/10.1111/jcpp.12381Return to ref 1 in article

Radez, J., Reardon, T., Creswell, C., Lawrence, P. J., Evdoka-Burton, G., & Waite, P. (2021). Why do children and adolescents (not) seek and access professional help for their mental health problems? A systematic review of quantitative and qualitative studies. In *European Child and Adolescent Psychiatry* (Vol. 30, Issue 2, pp. 183–211). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00787-019-01469-4

Sathyanarayana Rao, T., Asha, M., Ramesh, B., & Jagannatha Rao, K. (2008). Understanding nutrition, depression and mental illnesses. *Indian Journal of Psychiatry*, *50*(2), 77. https://doi.org/10.4103/0019-5545.42391

Schmidt, K., Cowen, P. J., Harmer, C. J., Tzortzis, G., Errington, S., & Burnet, P. W. J. (2015). Prebiotic intake reduces the waking cortisol response and alters emotional bias in healthy volunteers. *Psychopharmacology*, *232*(10), 1793–1801. https://doi.org/10.1007/s00213-014-3810-0

Schmidt, K., Cowen, P. J., Harmer, C. J., Tzortzis, G., Errington, S., & Burnet, P. W. J. (2015). Prebiotic intake reduces the waking cortisol response and alters emotional bias in healthy volunteers. *Psychopharmacology*, *232*(10), 1793–1801. https://doi.org/10.1007/s00213-014-3810-0

Selhub, E. M., Logan, A. C., & Bested, A. C. (2014). Fermented foods, microbiota, and mental health: Ancient practice meets nutritional psychiatry. In *Journal of Physiological Anthropology* (Vol. 33, Issue 1, p. 2). BioMed Central. https://doi.org/10.1186/1880-6805-33-2

Selhub, E. M., Logan, A. C., & Bested, A. C. (2014). Fermented foods, microbiota, and mental health: Ancient practice meets nutritional psychiatry. In *Journal of Physiological Anthropology* (Vol. 33, Issue 1). J Physiol Anthropol. https://doi.org/10.1186/1880-6805-33-2

Shah N (2007) Functional cultures and health benefits. *Inter-national Dairy Journal* **17**. 1262–1277.

Sheth M, Asudani A. (2014). Newer strategy to combat obesity amongst the bank employees of Urban Vadodara- Insights into its mechanism. World Journal of Pharmacy and Pharmaceutical Sciences, 4(2): 658-72.

Singh, M., Krishan Goel, N., Kumar Sharma, M., Kaur Bakshi, R., & Ravleen Kaur Bakshi, C. (2017). Prevalence of Depression, Anxiety and Stress among Students of Punjab University, Chandigarh. In *National Journal of Community Medicine│Volume* (Vol. 8, Issue 11). www.njcmindia.org

Terry, N., & Margolis, K. G. (2017). Serotonergic mechanisms regulating the GI tract: Experimental evidence and therapeutic relevance. *Handbook of Experimental Pharmacology*, *239*, 319–342. https://doi.org/10.1007/164_2016_103

Tuohy, K. M., Kolida, S., Lustenberger, A. M., & Gibson, G. R. (2001). The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructo-oligosaccharides – a human volunteer study. *British Journal of Nutrition*, *86*(3), 341–348. https://doi.org/10.1079/bjn2001394

Vankar, J. R., Prabhakaran, A., & Sharma, H. (2014). Depression and stigma in medical students at a private medical college. *Indian Journal of Psychological Medicine*, *36*(3), 246–254. https://doi.org/10.4103/0253-7176.135372

Wang, J., Um, P., Dickerman, B. A., & Liu, J. (2018). Zinc, magnesium, selenium and depression: A review of the evidence, potential mechanisms and implications. In *Nutrients* (Vol. 10, Issue 5). MDPI AG. https://doi.org/10.3390/nu10050584

Wang, X., & Gibson, G. R. (1993). Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *Journal of Applied Bacteriology*, *75*(4), 373–380. https://doi.org/10.1111/j.1365-2672.1993.tb02790.x

Yakunina, E. S., Rogers, J. R., Waehler, C. A., & Werth, J. L. (2010). College Students' Intentions to Seek Help for Suicidal Ideation: Accounting for the Help-Negation Effect. *Suicide and Life-Threatening Behavior*, *40*(5), 438–450. https://doi.org/10.1521/suli.2010.40.5.438