Project Title: Probing the Synergistic Effect of Pre-biotics & Iron Fortificants in Anemic Subjects	
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MATERIALS AND METHODS

The study will consist of determining the synergistic effect of pre-biotics and iron fortificants in anemic human subjects.

Study Design

A double blind, randomized controlled trial will be used for the purpose of this study.

Study Site

The study will be conducted in Islamabad and/or Lahore, depending on the availability of the required sample size with the desired profiles.

tudy Duration

The trial will last for 3 months.

Study Population

The study population will comprise of university going iron deficient female adults (age 18-25 years).

3.3.4.1 Inclusion and Exclusion Criteria

3.3.4.1.1 Inclusion Criteria

All willing anemic female adults without any chronic diseases such as diabetes or hypertension will be included in the study.

3.3.4.1.2 Exclusion Criteria

Married females or those with chronic diseases or those already taking iron and/or prebiotic supplements will be excluded from the study.

3.3.5. Sampling

3.3.5.1 Sampling Technique

The technique of convenience sampling will be used for the current study.

3.3.5.2 Sample Size

Each group will consist of 15 study subjects for the purpose of convenience and the total sample size will be 75.

3.3.6 Treatment Plan

For this phase of the study, 4 best treatment combinations of pre-biotics and iron fortificants will be chosen based on the results of animal trials while one group will be taken as control (Table 1).

Table 1: Treatment Plan for Anemic Women

GROUP	DIET PLAN
D_0	Control Group (Placebo)
D_1	Best Treatment Combination 1
D_2	Best Treatment Combination 2
D_3	Best Treatment Combination 3
D_4	Best Treatment Combination 4

Study participants will be randomly chosen to receive either the best treatment combination 1, 2, 3, 4 or the placebo for 12 weeks on daily basis.

3.3.7 Efficacy Trials

Up to three months, blood samples will be collected from overnight fasted women on monthly basis.

3.3.7.1 Hematological Analysis

The collected blood samples from all the groups will be subjected to hematological analysis. Blood complete picture including RBC indices such as Haemoglobin (Hb) levels and Hematocrit (Hr) levels & Leukocytes inlcuding Lymphocytes, Monocytes, Neutrophils and Basophils etc will be evaluated as per their respective protocols (Al Haj et al. 2011).

3.3.7.2 Iron Biomarkers

Blood serum biomarkers will be determined on the basis of analysis of obtained sera.

3.3.7.2.1 Serum Folate

Serum folate of the samples will be measured using the method mentioned by Shuaibi et al (Shuaibi et al. 2008).

3.3.7.2.2 Serum Iron

Concentration of serum iron concentration will be measured using Atomic Absorption Spectrophotometer (Wojciak et al. 2013).

3.3.7.2.3 Serum Ferritin

Ciba-Cornings Automated Chemiluminescence System's (ACS 180) commercially available kit will be employed to determine the levels of serum ferritin (Kazuaki et al. 2011).

3.3.7.2.4 Serum Transferrin

It will be measured as per the procedure menioned by Al-Buhairan and Oluboyede (Al-Buhairan and Oluboyede 2000).

3.3.7.2.5 Total Iron Binding Capacity (TIBC)

The standard formula for the determination of TIBC will be used;

TIBC = Transferrin x 24

3.3.7.2.6 Transferrin Saturation Fraction (TRSF)

TrSF will be calculated as per the following expression;

$$TrSF = \frac{\text{Serum Iron Concentration}}{\text{Total Iron Binding Capacity}} \times 100$$

3.3.7.3 Liver Function Tests (LFTs)

LFTs including AST (Aspartate Aminotransferase), ALT (Alanine Aminotransferase), ALP (Alkaline Phosphatase) and Total Bilirubin will be conducted (Basuny et al. 2009).

3.3.7.4 Renal Function Tests (RFTs)

RFTs including blood urea by GLDH-method and creatinine by Jaffe-method will be measured using their respective commercial kits (Jacobs 1996; Thomas 1998).

3.3.7.5 Immunoglobulins

Total Immunoglobulins will be determined using the technique of ELISA (Enzyme linked Immunosorbent Assay).

3.3.7.6 Statistical Analysis

In order to determine the level of significance, the statistical analysis of the obtained data will be done for the purpose of which, SPSS version 23.0 will be used. CRD will be used during the experiment and differences will be considered significant at P - value < 0.05 (Steel and Torrie 1980).

3.3.7.7 Ethical Considerations

The approval of the study will be taken from the Institutional Review Committee for Biomedical Research of the University of Veterinary & Animal Sciences, Lahore. Moreover, informed written consent will be obtained from all the study subjects. In addition to this, privacy and confidentiality of the data of all the study subjects will be ensured.