

Effects of synbiotic supplementation on energy and macronutrients homeostasis and muscle wasting of critical care patients: study protocol and a review of previous studies

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Study protocol

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Abstract

Background Among critically ill patients, regardless of the heterogeneity of disease state, an extreme and persistent dysbiosis occurs. Dysbiosis in critically ill patients may make them prone to hospital-acquired infections, sepsis, multi-organ failure (MOF), energy homeostasis disturbance, muscle wasting, and cachexia. Modulation of gut microbiota through synbiotics can be considered as a potential treatment for muscle wasting and macronutrients homeostasis disturbances.**Methods** This is a prospective, single center, double-blind; a parallel randomized controlled trial that aimed to evaluate the effects of synbiotic supplementation on energy and macronutrient homeostasis and muscle wasting in critical care patients. All eligible patients (20 subjects in each group) will receive standard hospital gavage as enteral nutrition through a nasogastric tube (NGT) in the 24-48h after admission. In the intervention group, patients will receive Lactocare (ZistTakhmir) capsules 500 mg every 12h for 14 days. Patients in the control group will receive a placebo capsule which contains only the sterile maize starch and is similar to synbiotic capsules. The synbiotic and placebo capsules will be given through nasogastric tube, separately from gavage, after feeding.**Discussion** Gut microbiota modulation through synbiotics is proposed to improve clinical prognosis and reduce infectious complications, ventilator dependency and ICU stay by improving energy and macronutrient homeostasis and reducing muscle protein catabolism.**Trials registration** The trial protocol has been approved in Iranian Registry of Clinical Trials at 2019-03-17. The registration reference is IRCT20190227042857N1.

Background

1.1 Gut microbiota, Dysbiosis and Critical Illness

The gut microbiota refers to the commensal microorganisms that reside in our gastrointestinal tract (GIT) with a symbiotic relationship(1). Gut microbiota has a significant role in the host metabolism and homeostasis(2, 3). A disturbance in this microbial community, which leads to an unhealthy state, is called dysbiosis(4). Over the past decade, emerging evidence revealed the role of intestinal dysbiosis in the pathogenesis of various conditions, such as infectious, immune and metabolic diseases(5), while in the critical illness, has not been studied as extensively. Among critically ill patients, regardless of the heterogeneity of disease state, an extreme and persistent dysbiosis occurs. The extreme dysbiosis occurred in critical care patients is due to the stress of critical illness, multiple antibiotics and additional pharmacological interventions, and highly processed enteral/parenteral nutrition (6, 7).

Dysbiosis in critically ill patients may make them prone to hospital-acquired infections, sepsis, multi-organ failure (MOF), energy homeostasis disturbance, muscle wasting, and cachexia(6, 8, 9).

1.2 Dysbiosis and Energy Homeostasis in Critical Illness

The majority of patients in Intensive care unit (ICU) have had a severe illness, trauma or major surgery, and accordingly they are unable to manage their nutritional demands. Although nutritional support is a daily practice in ICU, many patients still suffer from malnutrition due to lack of intake or uptake of nutrient(10). The prevalence of ICU malnutrition within developed and developing countries is reported as 50.8% and 78.1%, respectively(11). Malnutrition is independently associated with longer ICU stay, more ICU readmission, and a higher incidence of infections and risk of mortality(11). A greater degree of malnutrition is also associated with a higher risk of 28-day mortality(12). Malnutrition further tends toward acute or chronic loss of muscle bulk and function(13). The gut microbiota and its derived metabolites play an essential role in the absorption, storage, and consumption of energy derived from the diet(14, 15). Recent animal studies suggest that gut microbiota also regulates food intake and appetite by affecting hormones that influence metabolism and eating behavior(16, 17). Gut microbiota also affects metabolic function by modulating immune responses(18). Modulating gut microbiota by novel therapeutics such as prebiotics, probiotics or synbiotics can

eventually lead to the regulation of energy homeostasis and appetite. Recently Tuncay et al. showed that enteric formula with prebiotic content in neurocritical care patients was associated with a non-significant tendency to achieve a target dose of nutrition more frequent and earlier(19). Malik et al. also found that in the ICU patients receiving enteral formula supplemented with probiotics lead to a faster return of gut function(20).

1.3 Dysbiosis and muscle wasting in Critical Illness

Muscle wasting, characterized with a loss of muscle mass and strength, is associated with negative health outcomes such as functional disability, higher risk of infections, delayed recovery, poor life quality, and mortality(21). The gut microbiota has been recommended to influence muscle metabolism. The molecular mechanisms of this gut- muscle axis remain to be identified. Gut microbiota influences amino acid bioavailability and is a source of different metabolites such as conjugated linoleic acid, acetate and bile acids that modulate muscle metabolism. Various pathogen-associated molecular patterns (PAMPs) activate the transcription factor NF- κ B[1] through Toll-like receptors (TLRs) which causes muscle wasting. Gut microbiota also modulates production of proinflammatory cytokines which can induce muscle atrophy(8) (Figure 1).

Abbreviations: IL6, Interleukin 6; LPS, lipopolysaccharides; NF- κ B, Nuclear factor kappa light chain enhancer of activated B cells; PAMPs, pathogen-associated molecular patterns; SCFAs, short chain fatty acids; TLR4, Toll-like receptor 4; TNF α , tumor necrosis factor alpha.

Modulation of gut microbiota through pre, pro or synbiotics can be considered as a potential treatment for muscle wasting and cachexia. In mouse models of leukemia, restoring Lactobacillus species by oral supplementation with Lactobacillus reuteri 100-23 and Lactobacillus gasseri 311476 reduced inflammatory cytokines and expression of muscle atrophy makers(22). In another study, Bindels et al. showed that prebiotic supplementation in leukemic mice could contribute to delaying anorexia and fat mass sparing by inducing a metabolic shift in adipose tissue(23). In the mouse models of cancer cachexia administration of a synbiotic supplement including inulin-type fructans and live Lactobacillus reuteri 100-23 was associated with restoration of gut barrier and immune function thus reducing cachexia. It also prolonged survival (24). Varian et al. also showed that probiotic administration in leukemic mice could inhibit cachexia by reducing systemic inflammation (25).

1.4 Study Rationale

Considering extreme dysbiosis in critically ill patients and related energy and macronutrients homeostasis disturbance and muscle wasting, instigate us to evaluate the synbiotic supplementation effect on the elimination of such condition. To our knowledge this is the first study which investigates the synbiotic supplementation effect on muscle wasting of critically ill patients.

[1] Nuclear factor kappa light chain enhancer of activated B cells

Methods

2. Study Objectives

2.1 Primary Objective

Evaluating the effects of synbiotic supplementation on energy and macronutrient homeostasis and muscle wasting in critical care patients.

2.2 Secondary Objectives

Evaluating the effects of symbiotic supplementation on infectious complications and length of hospital and ICU stay in critical care patients.

3. Study Design

This is a prospective, single center, double-blind, parallel randomized controlled trial that will be conducted in Edalatian ICU, Emam Reza Hospital, Mashhad, Iran.

4. Selection and Enrollment of Participants

4.1 Inclusion criteria

Participants must meet all the inclusion criteria to participate in this study:

Adults aged 18-65 years, ICU admission, stable hemodynamic within 24-48 hour after admission, requiring enteral nutrition(EN) via nasogastric tube (NGT) feeding, not taking any kind of microbial cell preparations (pre, pro, synbiotic), and providing the written consent.

4.2 Exclusion criteria

All candidates meeting any of the exclusion criteria at baseline will be excluded from study participation:

Pregnancy and lactation, any contraindication of EN, any contraindication to placement of nasogastric feeding tube, receiving immunosuppressive treatment, radiotherapy or chemotherapy, hematologic diseases, acquired immune deficiency syndrome (AIDS), transplant recipient, known allergy to microbial cell preparations, cancer or autoimmune diseases, artificial heart valve or congenital heart valve disease.

4.3 Study enrollment procedure

Before the screening procedure, informed consent will be obtained from every participant who met the inclusion criteria. First, we will describe the purpose of the study, procedures involved, length of time the subject is suspected to participate, any possible disadvantages or discomforts, the benefits of the study for society and individuals, and person to contact for more question. We will also emphasize that participation is voluntary and refusal or withdraw will not cause any loss of benefits that they are entitled to receive. Then the participants or their legal guardian will read and sign the written form in two copies. If, because of the patient's lack of competence, the informed consent was obtained from his guardian, and during the study, the patient obtained the necessary qualification, consent will be regained.

4.4 Random allocation and blinding

After providing their written consent, patients are randomly allocated in a 1:1 ratio to the intervention or control group (A or B). The randomization will be performed through a stratified sequential randomization plan generated online. Randomization will be stratified by disease severity (APACHEII[1], 0-35 and 35-70). For allocation concealment we will use sealed opaque envelopes, inside each there is a carbon paper and the A or B card. To avoid the probable selection bias, we will write patient's name on the envelope before opening it. All patients, researchers, and medical staff will be blind about receiving either synbiotic or placebo capsules. An available third party, the secretary of ICU ward, will be aware that which of A or B is the synbiotic supplement. In case of discovering any complication associated with the intervention, the medical staff will refer to the secretary for details.

5. Study Interventions

5.1 Interventions, Administration, and Duration

All eligible patients will receive standard hospital gavage as EN through a nasogastric tube in the 24-48h after admission. According to the recent European Society of Parenteral and Enteral Nutrition (ESPEN) guideline on clinical nutrition in the ICU(26), continuous rather than bolus EN is preferred because it causes less diarrhea, but there is no difference in other outcomes. Another systematic review showed that bolus feeding is associated with lower aspiration rate and better calorie achievement(27). It also provides a greater stimulus for protein synthesis(28). Considering these data and the availability of bolus EN in our hospitals we applied this method. In the absence of indirect calorimeter, the simple weight-based equation of 20-25 Kcal/kg/day in acute flow phase and 25-30 Kcal/kg/day in anabolic flow phase is preferred to measure calorie requirements. For overweight and obese patients, ideal body weight: $0.9 \times \text{height (cm)} - 100$ (male) (or -106 (female)) is suggested as a reference weight (26). To avoid overfeeding, EN target will be prescribed within 3 days in high nutritional risk and 7 days in low nutritional risk patients according to modified NUTRIC[2] score. The flow charts in figure 2 and 3 will be used for initiation and continue of enteral nutrition.

In the intervention group, patients will receive Lactocare (ZistTakhmir) capsules 500 mg every 12h for 14 days. Each capsule contains *Lactobacillus casei* 1.5×10^9 CFU, *Lactobacillus acidophilus* 1.5×10^{10} CFU, *Lactobacillus rhamnosus* 3.5×10^9 CFU, *Lactobacillus bulgaricus* 2.5×10^8 CFU, *Bifidobacterium breve* 1×10^{10} CFU, *Bifidobacterium longum* 5×10^8 CFU, and *Streptococcus thermophilus* 1.5×10^8 CFU and FOS. The probiotics capsule will be given through nasogastric tube, separately from gavage, after feeding. Patients in the control group will receive a placebo capsule which contains only the sterile maize starch and is similar to probiotic capsules. The liquid preparations ready for gavage through NG tube are also similar in color and odor.

5.2 Handling of study intervention

The pharmaceutical company will provide synbiotic and placebo capsules in distinct boxes determined by A or B. Symbiotic capsules can be stored at room temperature for 2-3 weeks but the best condition for keeping this product is in the refrigerator at 2-8 ° C. Unused study products will be returned to the company supplying them.

5.3 Concomitant Interventions

- Antibiotics

It is common that critical care patients receive at least one antibiotic during their ICU stay. On the other hand, it is believed that antibiotics have bacteriostatic or bactericidal effects on both pathogenic and non- pathogenic bacteria. So, it is recommended that probiotics and antibiotics administration be separated at least by two hours(29).

- Opioids

Considering their analgesic and sedative properties, opioids are widely used in critical care patients. Opioids are believed to suppress the immune system and delay GI peristalsis. Delayed peristalsis can increase bacterial translocation out of the GI tract(30).

- H2 receptor blockers

Prevention of GIT stress ulcers, through H2 receptor blockers or proton-pump inhibitors, is common in critical care practice. Increase in GI acidity can cause some pathogenic bacteria to overgrowth (31).

- Catecholamines

It is believed that the elevated level of catecholamines in critical care patients, as prescribed exogenously beside endogenous production, can impair the immune system(32).

These drugs are routinely administered in critical care practice. So we will record and consider them as conflicting factors.

5.4 Adherence Assessment

As patients will receive the capsules through NGT by the researcher, adherence assessment is not required.

6. Study Procedures

6.1 Schedule of Evaluations

Schedule of Evaluations is shown in Table 1.

Table 1 Schedule of enrollment, intervention, and assessments in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; APACHE II, acute physiology and chronic health evaluation II; Cl, chloride; Cr, Creatinine; GCS, Glasgow coma scale; GRV, Gastric Residual volume; K, potassium; Mg, magnesium; Na, sodium; NUTRIC, nutrition risk in critically ill; P, phosphorus; PreAlb, pre-Albumin; SOFA, sequential organ failure assessment; TG, triglyceride.

6.2 Description of Evaluations

As it is shown in figure 2, calorie achievement goals are set according to the patients' modified NUTRIC score. In everyday visits, we will evaluate GI sign & symptoms (e.g. vomiting, diarrhea, abdominal distention) and GRV. If there is no sign or symptom of intolerance and GRV is less than 250 ml, EN will be increased by 10%. Otherwise, we will approach as figure 3. Energy homeostasis (calorie intake- estimated calorie requirement) will be recorded each day. Mid-arm circumference, which is an available anthropometric measurement tool, will be evaluated twice a week. As all patients receiving enteral nutrition should be monitored for some clinical and laboratory variables, we set our monitoring approach as Berger MM, et al., Monitoring nutrition in the ICU, Clinical Nutrition (2018). Concomitant medications, infectious complications and other adverse events will be recorded every day.

7. Safety Assessment

Despite the ample evidence supporting probiotics safety in critically ill populations, there are case reports of risks as well as suggested theoretical risks regarding probiotic administration. The most important is the risk of bacteremia and fungemia in the high-risk population, which may be associated with improper use and unintended contamination of central line catheters(32). To avoid bacteremia risk we will not include high-risk population, such as patients with recent major surgery, short bowel syndrome, heart valve disease or artificial heart valve and who are immunocompromised. We will also pay careful attention to the proper administration and hand washing protocols. Gene transfer and overstimulation of the immune system are other suggested theoretical risks which are not approved in human by any evidence(32).

8. Intervention Discontinuation

If intervention- related side effects exceed the level reported by previous studies, we will stop the intervention and present the results to the Ethics Committee of Mashhad University of Medical Sciences (MUMS) for further decision making.

9. Statistical Considerations

9.1 General design issues

Data will be analyzed with an intention to treat approach.

9.2 Sample size and randomization

We did not find any similar study which has evaluated our primary objectives. So, we considered one of the main secondary objectives to estimate the required sample size. Mahmoodpoor and co-workers reported the ICU stay in the two study groups as 18.6 ± 8 and 11.6 ± 6.3 days. Considering alpha error as 0.05 and a power of 80%, the required sample size with a 10% dropout was 20 patients in each group.

9.3 Outcomes

9.3.1 Primary outcomes

- Enteral feeding tolerance (abdominal examination and GRV measurement)
- Energy homeostasis (calorie intake- estimated calorie requirement)
- Protein catabolism (nitrogen balance)

Nitrogen balance is a measure of the net change in total body protein. It is the difference between nitrogen eliminated from the body and nitrogen ingested in the diet. A positive or neutral nitrogen balance shows that protein stores are increased or maintained, while a negative nitrogen balance indicates protein mass is decreasing. The practical method for estimating nitrogen balance supposes that total nitrogen loss is equal to urinary urea nitrogen excretion plus 4 g/day additional loss from non-urinary urea nitrogen, gastrointestinal and insensible losses(33, 34).

- Muscle protein degradation (3-methyl histidine (3MH) in 24h urine)

3MH is exclusively found in the muscle proteins and after protein degradation, it is rapidly excreted in the urine without further reutilization or metabolization. So, measuring urinary 3MH, after at least 1-day muscle-free diet, can be used as a biomarker of muscle protein breakdown(35, 36). ELIZA method will be used for 3MH detection.

- Muscle protein turnover (3MH/ Creatinine ratio in 24h urine)

Since the 24h urinary creatinine estimates the total pool of muscle proteins, muscle protein turnover can be calculated from 3MH/ creatinine excretion ratio(35).

- Lipolysis (free glycerol in serum)

Free glycerol is an important index of lipid metabolism. When the body uses stored fat as energy supply, glycerol and fatty acids are released into the circulation. The absence of glycerol kinase in the adipocyte decreases triacylglycerol resynthesis and supports hepatic gluconeogenesis. Free glycerol will be detected by enzymic colorimetric method.

- Glucose homeostasis (FBS, Insulin)
- Inflammatory status (CRP, Neutrophil/lymphocyte ratio (NLR))

NLR is an available measurable marker used to measure systemic inflammation.

- Dysbiosis status and luminal integrity (Endotoxin levels)

Intestinal gram-negative bacteria are the major source of lipopolysaccharides (LPS), which is referred to as endotoxin. In case of reduced intestinal barrier integrity due to dysbiosis, luminal endotoxins can enter the circulation(37). Endotoxin activity assay (EAA) will be used to determine endotoxin levels in whole blood.

- Clinical prognosis (APACHE and SOFA score)
- Nutritional status (NUTRIC score)

9.3.2 Secondary outcomes

- Infectious complications incidence
- Pressure ulcer incidence and its grade
- Ventilator- dependent days
- Length of ICU stay
- Length of hospital stay
- 28- Day mortality

9.4 Data analysis

Data will be analyzed with SPSS for windows version 11.5 and MedCalc Statistical Software version 18.11.3 (MedCalc Software bvba, Ostend, Belgium; <https://www.medcalc.org>; 2019). Descriptive (frequency, percentage, mean, standard deviation) and inferential analysis (student t test, paired sample t test, repeated measure ANOVA) will be performed. Any covariates will be controlled by ANCOVA or binary logistic regression. All tests will be two-tailed and a $p < 0.05$ will be considered as statistically significant.

10. Data Collection and Quality Assurance

Data gathering will be supervised by the primary investigator. Besides 10 percent of electronic data will be checked randomly with paper questionnaires and any discrepancies will lead to a 50% double checking of electronic data. Any outliers will be checked with patient medical records.

[1] Acute Physiology and Chronic Health Evaluation II

[2] Nutrition Risk In Critically Ill

Discussion

In the intestinal tract, gut microbiota controls different immune and endocrine functions(38). It has a major role in the absorption, storage ,and consumption of energy derived from the diet(14, 15). Outside the intestine, it also modulates cell metabolism, energy homeostasis, systemic inflammation, appetite and food intake (16, 38). On the other hand, a few clinical studies, modulating gut microbiota in critical care patients, demonstrated a faster return of gut function and earlier achievement of the nutritional target dose(19, 20). Therefore, we expect that our patients in the intervention group have a better enteric feeding tolerance and also more desirable energy homeostasis.

Animal studies has shown that modulation of gut microbiota by pre, pro or synbiotics can reduce cachexia and muscle mass sparing (22-25). The underlying mechanisms remain to be identified. Gut microbiota influences amino acid bioavailability and is a source of different metabolites such as conjugated linoleic acid, acetate and bile acids that modulate muscle metabolism. Gut microbiota is also a source of PMPS which activate the transcription factor NF- κ B

through Toll-like receptors (TLRs) and causes muscle wasting. Gut microbiota also modulates production of proinflammatory cytokines which can induce muscle atrophy(8). We expect that synbiotic intervention in critical care patients reduce muscle protein degradation and turnover. As malnutrition and muscle wasting in critical care patients are associated with negative health outcomes, gut microbiota modulation will improve clinical prognosis and reduce infectious complications, ventilator dependency and ICU stay.

Trial Status

Recruitment was started on 1 March 2019 and is estimated to be completed by October 2019. Recruitment was ongoing at the time of submission. This is the last protocol version (Number 4, 26 August 2019).

List Of Abbreviations

AIDS: Acquired Immune Deficiency Syndrome

ALT: Alanine aminotransferase

ANOVA: Analysis of variance

ANCOVA: Analysis of covariance

AST: Aspartate aminotransferase

APACHE II: Acute Physiology and Chronic Health Evaluation II

Cl: Chloride

Cr: Creatinine

CRP: C - reactive protein

MOF: Multi-Organ Failure

ELISA: Enzyme Linked ImmunoSorbent Assay

EAA: Endotoxin activity assay

EN: Enteral nutrition

ESPEN: European Society of Parenteral and Enteral Nutrition

GIT: Gastrointestinal tract

GCS: Glasgow coma scale

GRV: Gastric residual volume

ICU: Intensive Care Unit

K: Potassium

LPS: Lipopolysaccharides

3MH: 3-methy histidine

Mg: Magnesium

Na: Sodium

NGT: Nasogastric tube

NLR: Neutrophil\lymphocyte ratio

NUTRIC: Nutrition risk in critically ill

P: Phosphorus

PreAlb: pre-Albumin

PAMPs: Pathogen-associated molecular patterns

SOFA: Sequential Organ Failure Assessment

TG: Triglyceride

TLRs: Toll-like receptors

Declarations

- Ethics approval and consent to participate

Ethical approval was obtained from ethical committee of MUMS. The ethical approval code is IR.MUMS.MEDICAL.REC.1397.715. The informed consent will be obtained from all study participants or their legal guardian.

- Consent for publication

Not applicable

- Availability of data and material

The datasets generated and/or analyzed during the current study are not publicly available due to ethical considerations, but may be available from the corresponding author on reasonable request.

- Competing interests

No conflict of interest has been declared by the authors.

- Funding statement

This research will be funded by vice chancellery for research of Mashhad University of Medical Sciences (MUMS).

- Authors' contributions

NS and MS initially conceptualized and designed the study. MS, AS, MN and RR upgraded the protocol design and contributed in obtaining initial funding. Manuscript was written by NS and reviewed by all members. MKR was

responsible for design optimizing and statistical analysis.

- Acknowledgement

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Table

Table 1 Schedule of enrollment, intervention, and assessments in accordance with the Standard Protocol Recommendations for Interventional Trials (SPIRIT) guidelines.

STUDY PERIOD																	
	Enrolment	Allocation	Post-allocation														Close-out
Time point	Pre-allocation	Pre-intervention	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Enrollment																	
Informed consent form	x																
Eligibility	x																
Demographics	x																
Intervention			x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Assessments:																	
APACHEII, Modified NUTRIC, SOFA	x									x							x
GCS & Vital signs	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Energy homeostasis	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Abdominal examination																	According to Fig.3.
Fluid balance examination	x		x			x			x			x			x		x
GRV	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Mid arm circumference	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	
Infectious events	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Pressure ulcer	x																x
Mg,P,,Na,K,Cl	x					x			x			x			x		
Glucose	x									x							x
Insulin	x																x
AST, ALT ,TG, Urea	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	
PreAlb																	
Outcomes																	After 28 days
Concomitant medication																	

Figure 1

Dysbiosis and muscle wasting in critical illness. Abbreviations: IL6, Interleukin 6; LPS, lipopolysaccharides; NF-kB, Nuclear factor kappa light chain enhancer of activated B cells; PAMPs, pathogen-associated molecular patterns; SCFAs, short chain fatty acids; TLR4, Toll-like receptor 4; TNF α , tumor necrosis factor alpha.

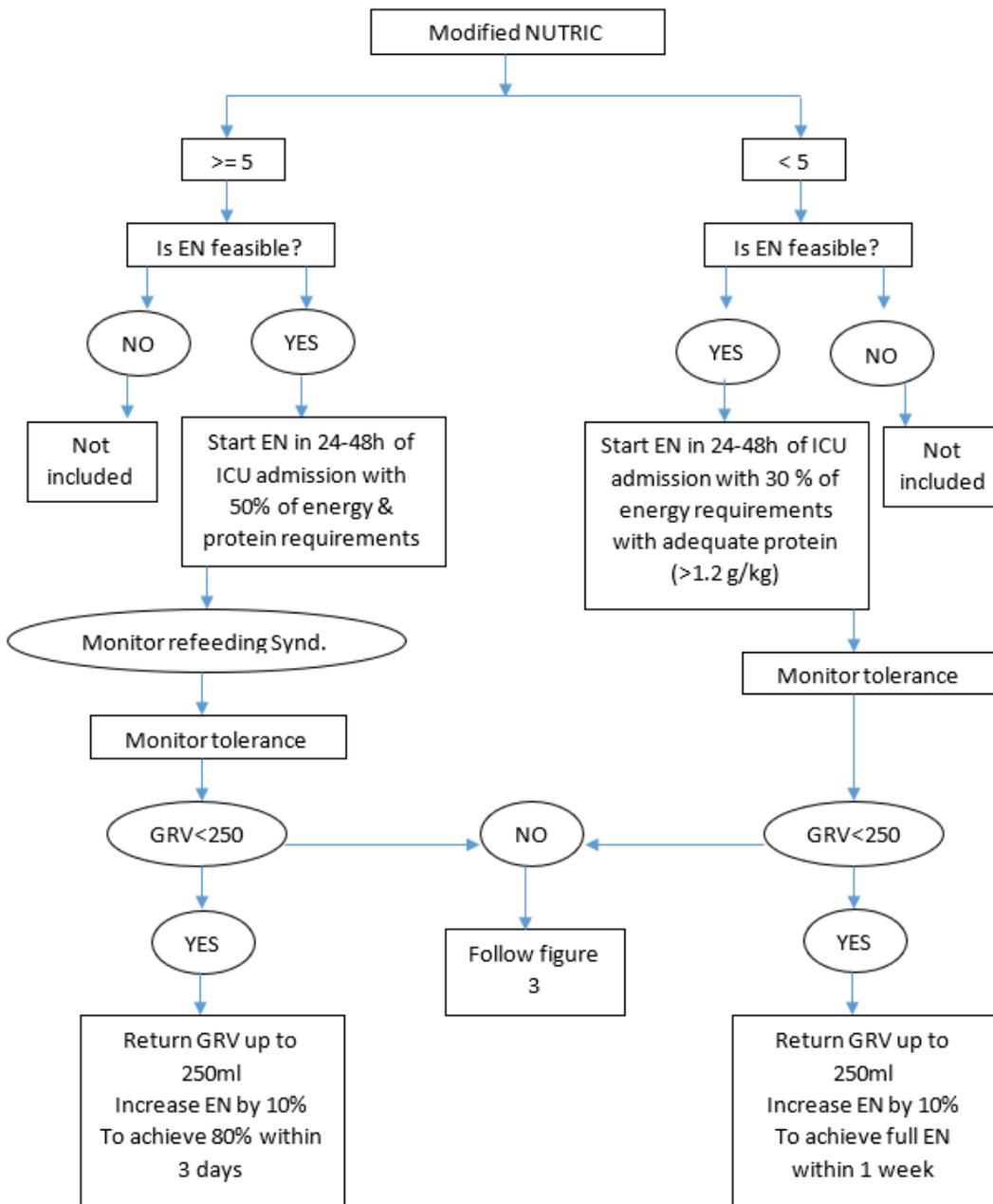


Figure 2

Dysbiosis and muscle wasting in critical illness. Abbreviations: IL6, Interleukin 6; LPS, lipopolysaccharides; NF-kB, Nuclear factor kappa light chain enhancer of activated B cells; PAMPs, pathogen-associated molecular patterns; SCFAs, short chain fatty acids; TLR4, Toll-like receptor 4; TNF α , tumor necrosis factor alpha.

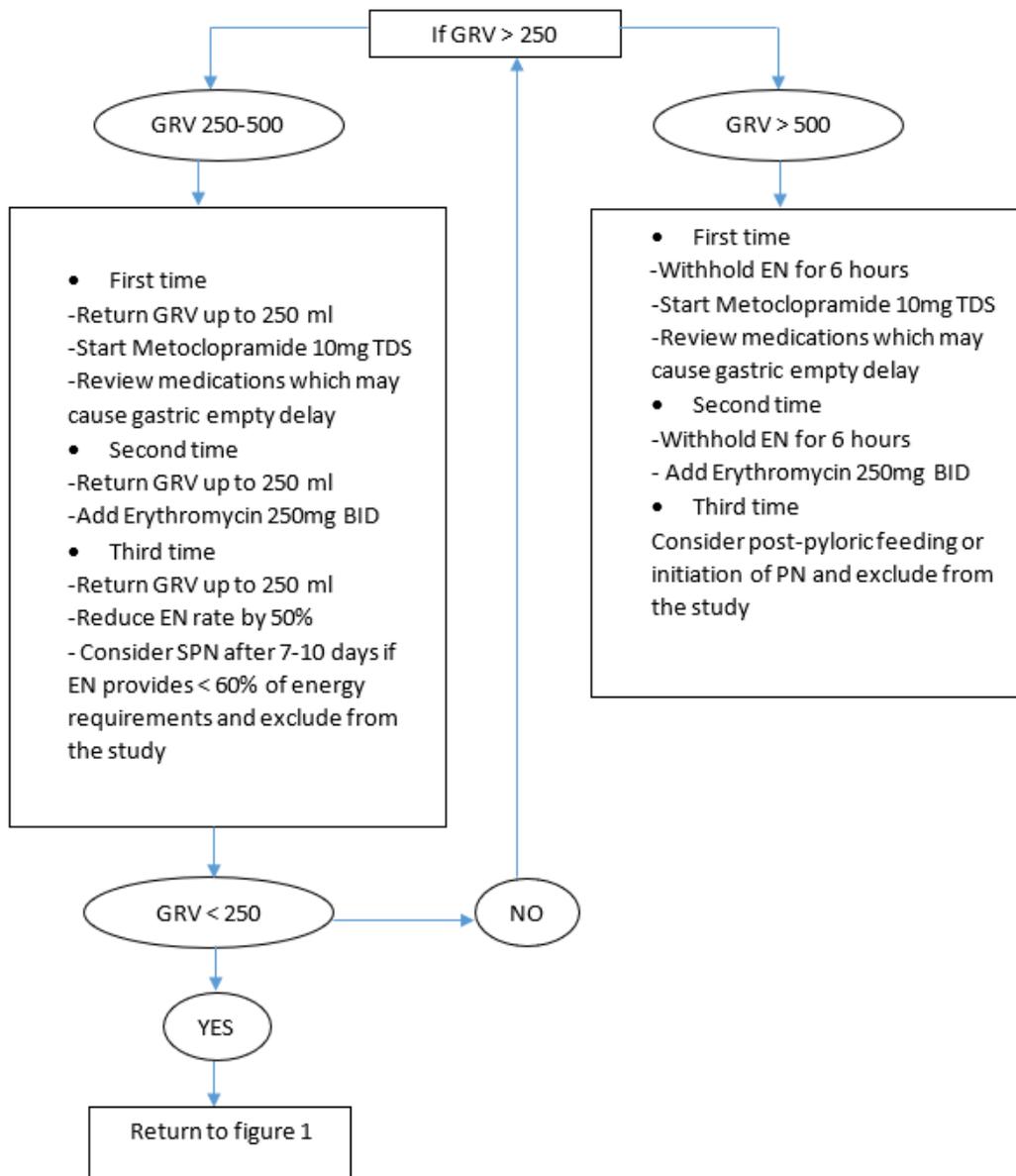


Figure 3

Flow Chart of GRV management Abbreviations: EN, enteral nutrition; GRV, gastric residuals volume; ICU, intensive care unit; ml, milliliter; NUTRIC, nutrition risk in critically ill; SPN, supplementary parenteral nutrition.

Supplementary Files

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- [SPIRIT.pdf](#)
- [CONSORT2010Checklist.pdf](#)