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Item	Halaman
Submitted to the journal (18-07-2024)	2
First revision (14-08-2024)	3
First revision submitted (03-09-2024) - Revisions and Amends - Revised version with highlights	4-33
Second revision (10-09-2024)	34
Proofread document received (16-09-2024) - Document from proofreading service	35-49
Second revision submitted (20-09-2024) - Revisions and Amends - Revised version with highlights	50-83
Third revision (01-10-2024)	84
Third revision submitted (14-10-2024) - Revisions and Amends - Revised version with highlight	85-113
Paper accepted (14-10-2024)	114-115
Paper published (28-10-2024) - Final paper	116-124

Submitted to the journal (18-07-2024)

From: Mental Health & Prevention <em@editorialmanager.com>
To: Alifiati Fitrikasari <fitrisutomo@yahoo.com>
Sent: Thursday, July 18, 2024 at 04:11:48 PM GMT+7
Subject: MHP-D-24-00222 - Confirming your submission to Mental Health & Prevention

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The Effect of Probiotic Supplementation on The Degree of Depression, Anxiety, and Stress in Cancer Patients with Chemotherapy

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First revision: Major revision (14-08-2024)

From: **Mental Health & Prevention** <em@editorialmanager.com>
Date: Wed, 14 Aug 2024, 11:37
Subject: Decision on submission to Mental Health & Prevention
To: Alifiati Fitrikasari <fitrisutomo@yahoo.com>

Manuscript Number: **MHP-D-24-00222**

The Effect of Probiotic Supplementation on The Degree of Depression, Anxiety, and Stress in Cancer Patients with Chemotherapy

Dear Mrs Fitrikasari,

Thank you for submitting your manuscript to Mental Health & Prevention. I have completed my evaluation of your manuscript, drawing on recommendations from three independent reviewers. The reviewers were split on their recommendations, and two were not confident that the identified issues could be addressed in revisions. However, I have decided to offer an opporutunity for consideration of your manuscript following major revision. Please note, I cannot gaurantee acceptance if you do decide to resubmit. The three reviewers offer excellent suggestions for strengthening the manuscript, below.

I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by **Sep 04, 2024**.

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Mental Health & Prevention values your contribution and I look forward to receiving your revised manuscript.

Kind regards,
Associate Professor Elizabeth Westrupp
Deputy Editor

Revised version received (03-09-2024)

From: **Mental Health & Prevention** <em@editorialmanager.com>
Date: Tue, 3 Sept 2024, 20:50
Subject: PDF for submission to Mental Health & Prevention requires approval
To: Alifiati Fitrikasari <fitrisutomo@yahoo.com>

This is an automated message.

The Effect of Probiotic Supplementation on The Degree of Depression, Anxiety, and Stress in Chemotherapy Patients: a Pilot Trial

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Kind regards,
Mental Health & Prevention

Revisions and Amends

Reviewer #1:

1. The study title should indicate that this is a pilot trial.

   The Effect of Probiotic Supplementation on The Degree of Depression, Anxiety, and Stress in Cancer Patients with Chemotherapy (Serotonin Biomarker Analysis): a Pilot Trial

2. Please note that scientific names such as "Lactobacillus rhomnosus" should be italicized as per convention.

   Already revised in the manuscript

3. The manuscript is in general need of language editing. Suggest a close proofreading ideally by a native English speaker. For example, "Previous studies have shown that cancer patients experience depression and anxiety, which usually appear in the early phase and during treatment" could be shortened to: "Cancer patients commonly experience depression and anxiety during treatment." "8 subjects are loss to follow up" is also incorrect, it should be "lost to follow up" and not "loss".

   The manuscript has been language edited.

4. In the methods section, please ensure that the trial is reported according to the CONSORT guidelines (citation: ncbi.nlm.nih.gov/pmc/articles/PMC2857832).

   Already revised based on the CONSORT guidelines. Please refer to method section.

5. The rationale for measuring serum serotonin levels should be at least briefly explained, and with appropriate supporting references. While serum serotonin levels can be indicative of systemic changes and are easily accessible for sampling, they may not accurately reflect the CNS serotonin levels. Serum serotonin is largely stored in platelets and can be influenced by factors unrelated to CNS serotonin activity.

   While CNS serotonin is already well-recognized to have direct implication in the pathophysiology of depression, anxiety, and stress, direct measurement of CNS serotonin is invasive and not feasible in clinical trial setting. Thus, in this study, serum serotonin levels were measures as a biomarker to assess the potential impact of probiotic supplementation on serotonin. We acknowledge that serum serotonin can be influenced by various peripheral factors and do not directly reflect CNS serotonin concentration. Though this is a limitation in the methodology, it is considered feasible and ethical of non-invasive

sampling in human subjects. Moreover, serum serotonin remains a valuable biomarker in studies exploring the gut-brain axis and the systemic effects of probiotic supplementation (Merkouris *et al.*, 2024). Previous studies reported that gut microbiota can modulate systemic serotonin levels, which in turn may influence CNS function through the gut-brain axis (Jenkins *et al.*, 2016; Potter *et al.*, 2023; Yano *et al.*, 2015). However, caution is required in interpreting these results, as the relationship between gut microbiota, serotonin levels, and psychological outcomes is complex and not fully understood.

6. How was the sample size determined? At least some elaboration is necessary.

   The sample size was determined using minimum sample of pilot clinical trial (**https://doi.org/10.1002/pst.185**).

7. Several aspects of the design need to be explained in greater detail. Please further explain the process of ensuring adherence to the probiotic regimen, as well as the exact procedures for randomization, allocation concealment, and blinding, to ensure reproducibility and replicability.

   Patients were randomly allocated to either the intervention or control group using block randomization with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order will be stored in a sealed envelope and will only be opened after the study is completed. The treatment code is inserted into the envelope and numbered according to the block order. Blinding was maintained by ensuring that the probiotics and placebo were identical in appearance, packaging, and administration. Probiotics and placebo were given in the form of capsules with the same color, size, and shapes. The placebo capsules were manufactured by the pharmaceutical laboratory. The placebo capsules contain the same additional substance as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to group assignments. Only pharmacist who knew the group assignments.

8. Please correct the stylistic and typo errors. It should be "54.46" and "49.08" rather than "54,46" and "49,08". Similarly, "0,710" should be "0.710".

   Already revised in the manuscript

9. There is no point reporting age with two decimal places. It is also more useful to report age using median and IQR.

   Already revised in the manuscript

10. The use of parametric tests (e.g., t-tests, ANOVA) on DASS scores can be problematic due to the ordinal nature of the data. Non-parametric tests (e.g., Mann-Whitney U, Wilcoxon signed-rank test) are more appropriate as they do not assume equal intervals.

    The statistic tests used in this study were Mann-Whitney test and Wilcoxon-signed rank test.

11. In Table 3, please change "Divorce" to "Divorced".

    Already revised in the manuscript

12. Regarding probiotics, the shift in the gut microbiota may be transient and temporary (citation: pubmed.ncbi.nlm.nih.gov/36986088) as several treatment trials for probiotics have failed to find significant alterations in gut microbiome; individuals may require longer duration of treatment to have therapeutic effects. This should be discussed.

Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence psychological symptoms, such as depression, anxiety, and stress through the gut-brain axis (Sabit *et al.*, 2023). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris *et al.*, 2024; Potter *et al.*, 2023; Ye *et al.*, 2022; Zhang *et al.*, 2023).

In this current study, the administration of probiotics over an 8-week period caused a significant decrease in the total DASS-42 scores in the intervention group ($p=0.001$), indicating an overall reduction in psychological distress. However, the reductions of each sub-scales (depression, anxiety, and stress) were not statistically significant ($p>0.005$). These finding suggest that, despite the beneficial effect on overall psychological symptoms, as measured by the total of DASS-42, the impact of probiotics on specific symptoms (depression, anxiety, and stress) may be more limited or require a longer duration on intervention to become significant.

This finding aligns with previous study which also implemented probiotic supplementation for eight weeks in a randomized controlled trial (RCT). It reported significant decrease in Beck Depression Inventory (BDI) scores in patients receiving probiotics compared to placebo (Akkasheh *et al.*, 2016). Some studies involving Lactobacillus species such as L. helveticus, L. rhamnosus, L. plantarum, and L. acidophilus used as probiotic ingredients showed a decrease in depression scale in some studies and showed no significant effect in other studies (Zhang et al.,

2023; Potter et al., 2023). However, other studies have shown only modest or inconsistent effects of probiotics on specific mood symptoms, especially in the short term intervention (Ng *et al.*, 2023). Another study found that supplementation with probiotics did not significantly reduce depression and anxiety levels in cancer and non-cancer patients (Ye et al., 2022).

Limitation

Another limitation of this study is the relatively short duration of the intervention. The 8-week period may not have been sufficient to observe significant changes in specific psychological symptoms, such as depression, anxiety, and stress. Studies suggest that the gut microbiota is a complex and dynamic ecosystem that may require longer periods of probiotic intervention to achieve significant alterations (Ng *et al.*, 2023).

Based on the considerations previously explained, future research should explore the effects of longer-term probiotic supplementation and investigate the optimal strains, dosages, and combination of probiotics that might result more consistent and significant impacts on gut microbiota and psychological health.

13. The discussion of results is also overly optimistic without sufficient consideration of the limitations.

    The discussion has already revised and considered several limitations

Reviewer #2:

1. The manuscript needs English polishing and appropriate reporting according to CONSORT statement.

   The manuscript has been language edited and reported according CONSORT statement.

2. In the abstract, conclusion should be edited. As it has been written with too much assertion. The conclusion should be limited to the results of the study and not exceed any more. It should only implicate the extraction of your own results.

   The study found that an 8-week probiotic supplementation regimen significantly decreased overall psychological symptoms as measured by the total DASS-42 scores. However, there

was no statistically significant changes in the specific subscales, such as depression, anxiety, and stress. In addition, even though the serum serotonin levels in the intervention group increased, this alteration was not statistically significant. These findings suggest that despite the role of probiotics in improving general psychological well-being in cancer patients undergoing chemotherapy, their impact on specific psychological symptoms and serotonin levels was still limited and require further investigation with longer intervention period.

3. The last paragraph of introduction is not well-written. The authors should imply to the necessity of their work much better.

Despite these promising findings, existing research has predominantly focused on the effects of probiotics on the physical side effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological symptoms in chemotherapy patients. In addition, the combination of probiotic used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) has never been studied to influence psychological disorder in human trial. Therefore, this study aims to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, with serum serotonin level as a potential biomarker for these effects.

4. It is exactly known whether the study design is interventional or randomized controlled trial. If it was carried out randomized manner, then why consecutive sampling method was applied? If it is an RCT then it should be mentioned everywhere especially in the title of the study.

   The sampling method has already revised. It did not use consecutive sampling. It used randomization sampling method.

5. In part 2.3.1. It is implied to an RCT design, but in the above paragraph, other method i.e. consecutive sampling is mentioned.

   The sampling method has already revised. It did not use consecutive sampling. It used randomization sampling method.

6. What were blocks stratified for?

Patients were randomly allocated to either the intervention or control group using block randomization with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order will be stored in a sealed envelope and will only be opened after the study is completed. The treatment code is inserted into the envelope and numbered according to the block order. Blinding was maintained by ensuring that the probiotics and placebo were identical in appearance, packaging, and administration. Both patients and investigators were blinded to group assignments. Only pharmacist who knew the group assignments.

7. Part 2.3.1. is written with future verbs!!

   Already revised into past tense verb

8. Was one week of antibiotic abstinence enough to allow someone to enter the study?

   The optimal abstinence period for antibiotics before entering study assessing probiotic and gut microbiota can vary depending on the type of antibiotic and patient's metabolism. Therefore, the abstinence period is calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days. Thus, a one-week abstinence period before study entry was reasonable to minimize the potential impact of residual antibiotics on gut microbiota and study outcomes.

9. Exclusion criteria seem not to be complete.

   Exclusion criteria were smoking or antibiotic use within one week before the intervention.

10. Figure one as the flowchart of the study is not acceptable. Figure 2 is enough.

    Figure one has been deleted

11. Whole verbs used in the study should be written in the simple past tense.

    Already revised

12. The method of analysis of normally and abnormally distributed data is not separately written. If it is an RCT, then, ANCOVA test should have been conducted.

    With the data we have, different age categories have the potential to be included as confounding factors, but the ANCOVA test cannot be performed properly due to the limited number of subjects.

13. In Table 3, plz merge numbers under 5 and avoid presenting the results in this way

    Already revised

14. **KT should be plus placebo

    Already revised

15. Tables 4 to 7 should be merged and presented in one Table. Further, as I stated above, ANCOVA test should be used for inter-group comparisons.

The tables have been merged, however the ANCOVA test cannot be performed properly due to the limited number of subjects.

16. According to the authors, "In this study, there was an increase in serotonin levels in the intervention group with probiotic administration, but it was not statically significant (p=0,382). (Table 8)". However, the authors have not paid attention to more noticeable changes in the control group than the intervention group. In most may be all parts, the changes are more observable in the control group!!

    We acknowledge that the changes in serotonin levels were more observable in the control group than the intervention group. It may be because of certain external factors, such as consumption of antiemetic medicine (ondansetron) by patients or dietary intake that includes amino acids. More explanation has been elaborated in the discussion part of the manuscript.

17. The authors have only discussed the mechanisms and nothing is discussed on the results of the study!

    The discussion part has been revised

18. The conclusion is very weak.

    The study found that an 8-week probiotic supplementation regimen significantly decreased overall psychological symptoms as measured by the total DASS-42 scores. However, there was no statistically significant changes in the specific subscales, such as depression, anxiety, and stress. In addition, even though the serum serotonin levels in the intervention group increased, this alteration was not statistically significant. In conclusion, the results of this pilot study are quite promising and should be continued with longer intervention period, larger sample sizes, and stricter inclusion and exclusion criteria.

Reviewer #3: This field is optioThe authors investigated Effects of "The Effect of Probiotic Supplementation on The Degree of Depression, Anxiety, and Stress in Cancer Patients with Chemotherapy". However, many points should be addressed in the revised version. This paper is novel due to the population studied and some significant results are reported which add to the field.

1. What has been the basis for the classification of the groups?

   If classification means randomization, it is as followed

Patients were randomly allocated to either the intervention or control group using block randomization with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order will be stored in a sealed envelope and will only be opened after the study is completed. The treatment code is inserted into the envelope and numbered according to the block order. Blinding was maintained by ensuring that the probiotics and placebo were identical in appearance, packaging, and administration. Probiotics and placebo were given in the form of capsules with the same color, size, and shapes. The placebo capsules were manufactured by the pharmaceutical laboratory. The placebo capsules contain the same additional substance as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to group assignments. Only pharmacist who knew the group assignments.

2. What is the justification in your choice of Probiotic dose?

   This study used the probiotic strains Lactobacillus rhomnosus Rossel 11 and Lactobacillus helveticus Rossel 52, which have generally been shown to reduce serotonin levels and symptoms of depression, either alone or in combination. The specific role of each probiotic, the optimal dose of probiotics, is still not well understood. The combination used in previous trials included Lactobacillus and Bifidobacterium, with study durations varying from 3-24 weeks.

3. The keywords should be taken from the Medical Subject Headings.

Keywords: probiotics, gut-brain axis, psychological, serotonin, chemotherapy

4. Please describe more about the novelty of manuscript in the introduction.

Existing research has predominantly focused on the effects of probiotics on the physical side effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological symptoms in chemotherapy patients. In addition, the combination of probiotic used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) has never been studied to influence psychological disorder in human trial. Therefore, this study aims to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, with serum serotonin level as a potential biomarker for these effects.

5. Is there a precise documentation of side effects following international standards? Please provide this information.

There was no any side effects documented because of probiotic supplementation in this study

6. Did not people study smoking and drug use? Do not use hypnotic drugs? Do you think these do not interfere with the study? How are they controlled?

The respondents used chemotherapy drugs but not antipsychotic drugs, such as benzodiazepine. It was already mentioned in the characteristics of the patient.

One drug that might influence serum serotonin level is ondansetron. We did not control it. Thus, we mentioned it as one of limitations of this study.

7. Blindness protocol and labeling methods should be described more comprehensively.

Patients were randomly allocated to either the intervention or control group using block randomization with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order will be stored in a sealed envelope and will only be opened after the study is completed. The treatment code is inserted into the envelope and numbered according to the block order. Blinding was maintained by ensuring that the probiotics and placebo were identical in appearance, packaging, and administration. Probiotics and placebo were given in the form of capsules with the same color, size, and shapes. The placebo capsules were manufactured by the pharmaceutical laboratory. The placebo capsules contain the same additional substance as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and

investigators were blinded to group assignments. Only pharmacist who knew the group assignments.

8. No information about the physical activity records.

Yes, we did not record the physical activities of the respondents. Thus, we mentioned it as one of the study limitations.

9. The table result is unclear. Please state the changes between the two groups in the variables examined, also β (95% CI).

The table has already been revised.

10. In the discussion, refer to the new article published in this field." The Effects of Probiotic Supplementation on Opioid-Related Disorder in Patients under Methadone Maintenance Treatment Programs". And https://doi.org/10.1016/j.ctim.2020.102361

Those articles have been cited in the manuscript.

11. What was the composition of the placebo?

The placebo capsules contain the same additional substance as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results.

12. Please describe the statistical analyses in more detail. For example, did you adjust the outcome variable measured in the follow-up measurements for the baseline value of the outcome (according to the equation $Yt = \beta0 + \beta1 * X + \beta1 * Yt0$, where Yt = the outcome measured in the two follow-up measurements, X = treatment variable, β1 = overall treatment effect, and Yt0 = outcome variable measured in the baseline measurement)? Please indicate the parameters you have adjusted for in your linear regression analysis. It is well acceptable that an appropriate significance level α, such as 0.05, is pre-specified to guarantee the probability of incorrectly rejecting a single test of null hypothesis (H0) no larger than α. However, there are many situations where more than one or even a large number of hypotheses are simultaneously tested, which is referred to as multiple comparisons. Because you are testing many different hypothesis simultaneously ("multiple comparisons"), proper adjustment of statistical inference is required.

With the data we have, different age categories have the potential to be included as confounding factors, but the ANCOVA test cannot be performed properly due to the limited number of subjects.

13. Identify the primary and secondary outcomes.
nal. If you have any additional suggestions beyond those relevant to the questions above, please number and list them here.

The primary outcome was the change in depression, anxiety, and stress levels, measured using the Depression-Anxiety-Stress Scale-42 (DASS-42) at baseline and after 8 weeks of intervention. Secondary outcome included changes in serum serotonin levels for 8 weeks, measures by enzyme-linked immunoassay (ELISA).

Revised version

ABSTRACT

Background: Psychological disorders, including depression, anxiety, and stress, are prevalent among cancer patients undergoing chemotherapy. Probiotic has been investigated as a potential supplementation to modulate the gut-brain axis and improve psychological symptoms through mechanisms such as serotonin regulations. However, the study that specifically examined the effects of probiotics on psychological symptoms in chemotherapy patients is still limited.

Methods: This randomized, double-blind, placebo-controlled pilot trial was conducted at the outpatient clinic of [redacted], in 2023. Sixty-one cancer patients undergoing chemotherapy were enrolled and randomized into intervention (n=30) and control (n=31) groups. The intervention groups received probiotics (*Lactobacillus rhamnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) twice daily for 8 weeks. The primary outcome were changes in depression, anxiety, and stress levels measured by the Depression-Anxiety-Stress Scale-42 (DASS-42). Secondary outcomes included serum serotonin levels.

Results: The intervention group showed a significant reduction in total DASS-42 scores ($p=0.001$) after 8 weeks, indicating an overall decrease in psychological distress. However, changes in individual subscales for DASS-42 were not statistically significant ($p>0.05$). Serum serotonin levels increased in the intervention group, but the change was not statistically significant ($p=0.382$).

Conclusion: While probiotic supplementation significantly reduced overall psychological symptoms, its impact on specific symptoms and serotonin levels was limited. Future research should explore longer intervention periods, larger sample sizes, and control for external factor more rigorously to better understand the therapeutic impact of probiotics in cancer patients undergoing chemotherapy.

Keywords: probiotics, gut-brain axis, psychological, serotonin, chemotherapy

1. Introduction

Psychological disorders are increasingly prevalent among patients diagnosed with cancer, with conditions such as delirium, depression, adjustment disorders, anxiety, sexual dysfunction, and sleep disorders affecting 30%-40% of this population. The incidence of psychiatric disorders is even higher among those in advanced stages of cancer. In Indonesia, 34.4% of cancer patients suffer from depression, with the prevalence rising as the severity and duration of the disease increase. Additionally, a study in one Indonesian hospital reported that 23% of patients undergoing chemotherapy experienced depression, while 40% suffered from anxiety. Despite the significant impact of these conditions on patients' quality of life, psychiatric disorders in cancer patients are often underdiagnosed and inadequately treated, leading to further deterioration in their overall well-being (Mastan *et al.*, 2024; Ostovar *et al.*, 2022; Setiyarini *et al.*, n.d.).

Chemotherapy has been shown to disrupt the balance of gut microbiota, a condition known as gut dysbiosis (Deleemans *et al.*, 2019). This disruption can lead to a reduction in the diversity and number of commensal bacteria, which in turn can negatively affect mood and cognitive function (Deleemans *et al.*, 2019; Maddern *et al.*, 2023). Research has demonstrated that changes in microbiota composition can influence the development of psychological symptoms, such as depression and anxiety (Deleemans *et al.*, 2019; Maddern *et al.*, 2023). Animal studies have further demonstrated that gut microbiota plays a critical role in regulating pathway associated with depression (Deleemans *et al.*, 2019), highlighting the potential of gut microbiota as a therapeutic target for psychological disorders in cancer patients.

Given that psychological disorders are linked to low serotonin levels – a condition that can be exacerbated by gut dysbiosis – targeting the gut microbiota through oral probiotics presents a promising therapeutic approach (Merkouris *et al.*, 2024; Zhang *et al.*, 2023). Probiotics have traditionally been used to mitigate gastrointestinal side effects of chemotherapy, such as nausea

and vomiting (Vivarelli *et al.*, 2019). Recent studies suggest that probiotics may improve psychological well-being by influencing neurotransmitter pathways, including serotonin, which is closely associated with mood regulation (Merkouris *et al.*, 2024; Zhang *et al.*, 2023). Specifically, probiotics containing such as *Lactobacillus rhamnosus* and *Lactobacillus helveticus* have been shown in animal studies to reduce symptoms of depression, enhance cognitive function, and balance key neurochemicals, including serotonin, epinephrine, and brain-derived neurotrophic factor (BDNF) (Deleemans *et al.*, 2019; Ye *et al.*, 2022).

Despite these promising findings, existing research has predominantly focused on the effects of probiotics on the physical side effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological symptoms in chemotherapy patients. In addition, the combination of probiotic used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) has never been studied to influence psychological disorder in human trial. Therefore, this study aims to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, with serum serotonin level as a potential biomarker for these effects.

2. Method

This study was a randomized, double-blind, placebo-controlled pilot trial conducted at Outpatient Clinic of [redacted] in 2023. The trial was designed to assess the effect of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, also serotonin levels as a biomarker.

2.1 Participants

Participants included cancer patients who were undergoing chemotherapy in Dr. Kariadi Hospital. Inclusion criteria required patients aged 18-76 years. Exclusion criteria were smoking or antibiotic use within one week before the intervention.

The optimal abstinence period for antibiotics before entering study assessing probiotic and gut microbiota can vary depending on the type of antibiotic and patient's metabolism. Therefore, the abstinence period is calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days (Armstrong, 2020). Thus, a one-week abstinence

period before study entry was reasonable to minimize the potential impact of residual antibiotics on gut microbiota and study outcomes.

2.2 Sample Size

The sample size was determined using minimum sample of pilot clinical trial (Julious, 2005). A total of 61 patients were enrolled and randomized into the intervention (n=30) and control (n=31) groups, with an allocation ration of 1:1.

2.3 Randomization and Blinding

Patients were randomly allocated to either the intervention or control group using block randomization with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order will be stored in a sealed envelope and will only be opened after the study is completed. The treatment code is inserted into the envelope and numbered according to the block order. Blinding was maintained by ensuring that the probiotics and placebo were identical in appearance, packaging, and administration. Probiotics and placebo were given in the form of capsules with the same color, size, and shapes. The placebo capsules were manufactured by the pharmaceutical laboratory of [redacted]. The placebo capsules contain the same additional substance as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to group assignments. Only pharmacist who knew the group assignments.

2.4 Intervention

The treatment will be given according to the arrival of the research subject. The intervention group received probiotics (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52 at a dose of $2x10^9$ CFU) twice a day for 8 weeks.

2.5 Outcomes

The primary outcome was the change in depression, anxiety, and stress levels, measured using the Depression-Anxiety-Stress Scale-42 (DASS-42) at baseline and after 8 weeks of intervention. Secondary outcome included changes in serum serotonin levels for 8 weeks, measures by enzyme-linked immunoassay (ELISA).

2.6 Research Instruments

To assess the primary outcomes, the following instruments were used:

1) Depression-Anxiety-Stress Scale-42 (DASS-42)

   The DASS-42 is a 42-item questionnaire used to measure the severity of depression, anxiety, and stress in participants. The questionnaire consists of 42 assessment statements (table 1) to assess depression (14 statements), anxiety (14 statements), and stress (14 statements). Participants were asked to score each statement with 0 = never, 1 - sometimes,2 = often, and 3 = very often. Subscales were then summed to determine the depression, anxiety, and stress scales (table 2).

Table 1. Statements for depression, anxiety, and stress subscales

Subscale	**Statement Number**
Depress	3, 5, 10, 13, 16, 17, 21, 24, 26, 31, 34, 37, 38, 42
Anxiety	2, 4, 7, 9, 15, 19, 20, 23, 25, 28, 30, 36, 40, 41
Stress	1, 6, 8, 11, 12, 14, 18, 22, 27, 29, 32, 33, 35, 39

Table 2. DASS-42 Interpretation

	Depression	**Anxiety**	**Stress**
Normal	0-9	0-7	0-14
Mild	10-13	8-9	15-18
Moderate	14-20	10-14	19-25
Severe	21-27	15-19	26-33
Very Severe	≥28	≥20	≥34

Meanwhile, to assess secondary outcome, the following instruments were used:

2) Serotonin measurement

Serum serotonin levels were measured using an enzyme-linked immunosorbent assay (ELISA), following the manufacturer's protocol. Blood samples were collected at baseline and after the intervention, and results were read using a microplate reader (ELx800).

2.7 Statistical Methods

Data were analyzed using intention-to-treat principles. Continuous variables (age, DASS score, serotonin level) were summarized using mean ± standard deviation, while categorical variables (gender, marital status, education, iccupation, history of psychiatric illness, history of psychiatric treatment, and duration of cancer diagnosis) were presented as frequencies and percentages. The Saphiro-Wilk test was used to assess the normality of data distribution. Between-group comparison were conducted using the Mann-Whitney test for continuous variables and Chi-square test for categorical variables. Within-group comparisons were analyzed using the Wilcoxon-signed rank test. A p=value < 0.05 was considered statistically significant.

2.8 Ethics

The study was registered at the Indonesian Clinical Research Registry (INA-CRR) with registration number 042024030706474KRGNHZ and approved by the Health Research Ethics Committee of [redacted] (No. 1496/EC/KEPK-RSDK/2023). All participants provided written informed consent. The trial was conducted according to the principles of the Declaration of Helsinki.

3. Results

3.1 Sample characteristics

This study involved 61 cancer patients who met the inclusion and exclusion criteria. The study sample was divided into 2 groups by randomization, the intervention group and the control group. A total of 2 research subjects from the intervention group died, 4 subjects are hospitalized during the trial, 3 subjects did not come to control for treatment and 8 subjects are loss to follow up so the intervention group consisted of 13 subjects. Meanwhile, 2 subjects from the control group died,

1 subject dropped out from taking medicine, 3 subjects hospitalized, 4 subjects did not come to control for treatment and 15 subjects are loss to follow up so the control group consisted of 13 subjects. (Figure 1).

Table 3 shows the characteristics of the study sample in each intervention group and control group. In this study sample, the mean age in the intervention and control groups was 54,46 and 49,08 years, respectively. The number of male and female patients was approximately equal in the intervention and control groups. Most of the study sample were married (84,6%), had the highest education level of high school (30.7%), and worked (65%). All samples had no history of psychiatric treatment or previous psychiatric diagnosis. There were no significant differences in age, gender, marital status, education, employment status, and duration of cancer diagnosis.

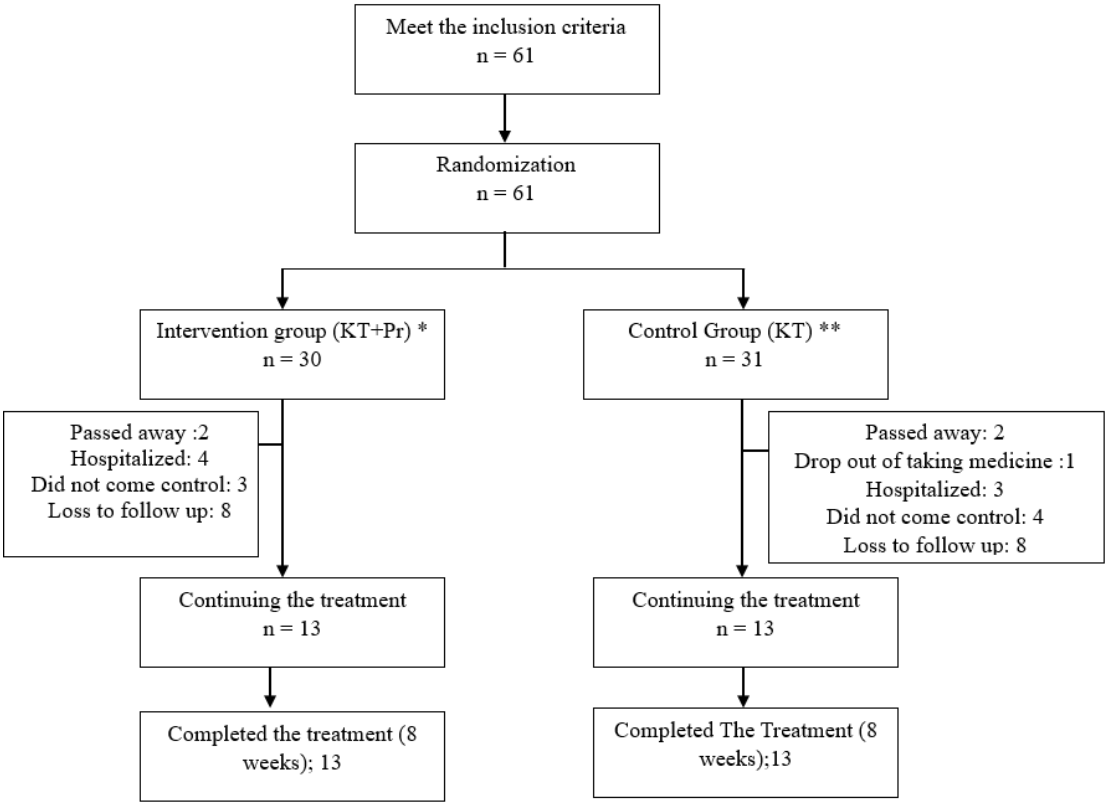

Figure 1. Research CONSORT Diagram. This study involved 61 cancer patients, randomizing into an

intervention group and a control group.

Description:

*KT + Pr = Chemotherapy + Probiotics

**KT = Chemotherapy+placebo

Table 3. Characteristics of the research sample

Variables	**Intervention (n=13)**	**Control (n=13)**	**p-value***
Age			
Mean ± SD	54 ± 7.88	49 ± 17.06	0.079
Median (Min-Max)	54 (43-68)	54 (23-76)	
	Frequency (%)		
Variables	**Intervention (n=13)**	**Control (n=13)**	**P value**
Gender			
Male	6 (46.2%)	6 (46.2%)	1.000¥
Female	7 (53.8%)	7 (53.8%)	
Marital Status			
Not Married	1 (7.7%)	2 (15.4%)	0.703¥
Married	11 (84.6%)	11 (84.6%)	
Divorced	1 (7.7%)	0 (0%)	
Highest Education			
Elementary School	1 (7.7%)	1 (7.7%)	0.710‡
Junior High School	2 (15.4%)	4 (30.8%)	
Senior High School	5 (38.5%)	3 (23.1%)	
Bachelor	4 (30.8%)	4 (30.8%)	

Not in school	1 (7.7%)	1 (7.7%)	
Jobs			
Working	8 (61.5%)	9 (69.2%)	0.500
Not Working	5 (38.5%)	4 (30.8%)	
History of psychiatric treatment (including benzodiazepine)			
Yes	0	0	. [e]
No	13 (50%)	13 (50%)	
Psychiatric diagnosis			
Yes	0	0	. [e]
No	13 (50%)	13 (50%)	
Duration of psychiatric treatment			
Yes	0	0	. [e]
No	13 (50%)	13 (50%)	
Duration of cancer diagnosis			
3 – 6 months	2 (15.4%)	4 (30.8%)	0.518[ǂ]
6 months – 1 year	5 (38.5%)	4 (30.8%)	
1 – 5 years	5 (38.5%)	4 (30.8%)	
>5 years	1 (7.7%)	1 (7.7%)	

[ǂ] Mann-Whitney; [¥] Chi-Square ;[e] Fischer-exact test; Not measurable because n=0

*P value <0.05 is considered as statistically significant

3.2 Effect of Probiotics on Depression, Stress, and Anxiety

The results showed insignificant decrease in depression scores (p = 0.317), anxiety (p = 0.914), stress (p = 0.581) , while the result show significant decrease in total DASS-42 scores (p = 0.001) in the intervention group after administering probiotics for 8 weeks (Table 4). While from the comparison of the control group with the intervention group, there were insignificant difference between the scores depression, anxiety and stress but the scores of total DASS-42 in the

intervention group and the control group had significant difference after administering probiotics for 8 weeks (p=0.048) (Table 4).

Table 4. Comparison of depression scores of the intervention group before and after the intervention

DASS	Group		p
	Intervention	Control	
Pre	19.00 ± 7.10	13.69 ± 7.06	0.068§
Post	17.38 ± 6.48	11.15 ± 6.83	0.048‡*
p	0.001¶*	0.002†*	0.207§
Depression			
Pre	5.89 ± 3.20	6.31 ± 3.77	0.658§
Post	5.46 ± 3.05	4.69 ± 2.78	0.508§
p	0.317†	0.010†*	0.058‡
Anxiety			
Pre	5.23 ± 3.86	5.15 ± 8.16	0.188‡
Post	5.46 ± 4.05	3.54 ± 4.82	0.055‡
p	0.914†	0.024†*	0.081‡
Stress			
Pre	6.15 ± 3.02	9.15 ± 5.54	0.099§
Post	6.46 ± 2.33	6.92 ± 4.27	0.735§
p	0.581†	0.007†*	0.003‡

* Statistically significant ($p < 0.05$); ‡ Mann-Whitney; § Independent t; ¶ Paired t; † Wilcoxon

3.1 Effect of Probiotics on Serotonin

In this study, there was an increase in serotonin levels in the intervention group with probiotic administration, but it was not statistically significant (p=0.382). (Table 5)

Table 5. Comparison of serotonin levels of intervention and control groups

Serotonin	Group		p
	Intervention	Control	
Pre	98.85 ± 125.22	145.77 ± 199.78	0.798‡
Post	104.15 ± 195.69	161.38 ± 175.37	0.012‡*
p	0.382†	0.087†	
Difference	5.31 ± 77.48	15.62 ± 66.20	0.048‡*

*Statistically significant ($p < 0.05$); ‡ Mann-Whitney; † Wilcoxon

4. Discussion

Cancer patients undergoing chemotherapy frequently experience significant psychological distress including depression, anxiety, and stress, which adversely affect their quality of life (Ostovar *et al.*, 2022). Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence psychological symptoms, such as depression, anxiety, and stress through the gut-brain axis (Sabit *et al.*, 2023). A systematic review also reported the beneficial effects of probiotic supplementation on Hamilton Depression Rating Scale (HAMD) in patients with psychiatric disorders (Amirani *et al.*, 2020). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris *et al.*, 2024; Potter *et al.*, 2023; Ye *et al.*, 2022; Zhang *et al.*, 2023).

In this current study, the administration of probiotics over an 8-week period caused a significant decrease in the total DASS-42 scores in the intervention group ($p=0.001$), indicating an overall reduction in psychological distress. However, the reductions of each sub-scales (depression, anxiety, and stress) were not statistically significant ($p>0.005$). These finding suggest that, despite the beneficial effect on overall psychological symptoms, as measured by the total of DASS-42, the impact of probiotics on specific symptoms (depression, anxiety, and stress) may be more limited or require a longer duration on intervention to become significant.

Chemotherapy, radiotherapy, and immunotherapy have toxicity effects that can lead to changes in gut microbiota, reduction of gut commensal bacteria, and inflammation of the gastrointestinal tract. Research has found a bidirectional relationship between the digestive system and the nervous

system (gut-brain axis) (Deleemans *et al.*, 2019; Ichim *et al.*, 2018; Maddern *et al.*, 2023; Vivarelli *et al.*, 2019). Gut dysbiosis can lead to increased gut permeability, allowing toxins to enter the bloodstream and cause the activation of pro-inflammatory cytokines (IL-6, II-1b, TNF-a, and C-reactive protein (CRP)) and hyperactivation of the hypothalamic-pituitary axis (HPA). These inflammatory conditions then lead to decreased levels of serotonin (5-HT) and brain-derived neurotrophic factor (BDNF). Both can cause psychological and cognitive changes such as anxiety and depression, fatigue, memory impairment, and impaired decision-making (Lu *et al.*, 2022; Maddern *et al.*, 2023; Merkouris *et al.*, 2024; Sabit *et al.*, 2023).

The mechanism of psychological disturbance discussed in this study is related to chemotherapy-induced gut dysbiosis with serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p=0.0382$). Interestingly, the control group, which did not receive probiotic, demonstrated more pronounced changes in serotonin levels. There are certain external factors that may have influences these results.

One factor is the potential consumption of ondansetron by patients. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta *et al.*, 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels, potentially explaining the more pronounced changes observed in the control group.

In addition, dietary intake that includes amino acids such as tryptophan, a precursor of serotonin, could have contributed to variations in serotonin levels (Jenkins *et al.*, 2016; Mohajeri *et al.*, 2015). Tryptophan-rich foods can increase serotonin synthesis. However, dietary habits were not controlled in this study, thus, the differences in dietary intake among participants might have affected the results. Future studies should aim to control for the medications and dietary intake more rigorously.

The increase in serotonin is one of several different mechanisms involved in the improvement of depression, anxiety, and stress symptoms from probiotic use. In addition to serotonin production, probiotics may reduce symptoms of depression and anxiety through several different mechanisms, including decreasing stress-induced HPA responses, lowering cortisol levels, increasing neurotransmitter synthesis (GABA, dopamine, noradrenaline, melatonin, histamine,

and acetylcholine), stimulating the production of gut neuropeptides [glucagons like peptide-1 (GLP-1) and tyrosine (PYY)], improving the gut barrier, increasing BDNF production, and decreasing the release of pro-inflammatory cytokines and increasing anti-inflammatory cytokines (Lu *et al.*, 2022; Sabit *et al.*, 2023; Zhang *et al.*, 2023).

Gut-brain axis and serotonin production are influenced by a complex of factors beyond just probiotic supplementation, for example stress levels, diet, and physical activity (Lou *et al.*, 2023; Madison and Kiecolt-Glaser, 2019; Mohajeri *et al.*, 2015). The influence of these variables might have differed between the control and intervention groups, contributing to the outcomes.

4.1 The study limitation

This study has several limitations, such as the use of serum serotonin as biomarker. While CNS serotonin is already well-recognized to have direct implication in the pathophysiology of depression, anxiety, and stress, direct measurement of CNS serotonin is invasive and not feasible in clinical trial setting. Thus, in this study, serum serotonin levels were measures as a biomarker to assess the potential impact of probiotic supplementation on serotonin. We acknowledge that serum serotonin can be influenced by various peripheral factors and do not directly reflect CNS serotonin concentration. Though this is a limitation in the methodology, it is considered feasible and ethical of non-invasive sampling in human subjects. Moreover, serum serotonin remains a valuable biomarker in studies exploring the gut-brain axis and the systemic effects of probiotic supplementation (Merkouris *et al.*, 2024). Previous studies reported that gut microbiota can modulate systemic serotonin levels, which in turn may influence CNS function through the gut-brain axis (Jenkins *et al.*, 2016; Potter *et al.*, 2023; Yano *et al.*, 2015).

Another limitation of this study is the relatively short duration of the intervention. The 8-week period may not have been sufficient to observe significant changes in specific psychological symptoms, such as depression, anxiety, and stress. Studies suggest that the gut microbiota is a complex and dynamic ecosystem that may require longer periods of probiotic intervention to achieve significant alterations (Ng *et al.*, 2023). Another study also reported the beneficial effects were seen on symptoms of depression after probiotic supplementation for 12 weeks to patients under methadone maintenance treatment programs (MMTP) (Molavi *et al.*, 2022).

Additionally, the study did not control for physical activity, dietary habits, symptomatic medications, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of the patients.

Based on the considerations previously explained, future research should explore the effects of longer-term probiotic supplementation and control for external factors more rigorously, perhaps through more detailed dietary assessments, physical activity log, and closer monitoring of medication use.

5. Conclusions

The study found that an 8-week probiotic supplementation regimen significantly decreased overall psychological symptoms as measured by the total DASS-42 scores. However, there was no statistically significant changes in the specific subscales, such as depression, anxiety, and stress. In addition, even though the serum serotonin levels in the intervention group increased, this alteration was not statistically significant. In conclusion, the results of this pilot study are quite promising and should be continued with longer intervention period, larger sample sizes, and stricter inclusion and exclusion criteria.

Conflicts of Interests:

The author declares no organizational involvement or financial or financial involvement in the material discussed in this article.

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Data availability statement

The data obtained from this study were ethically protected by the Health Research Ethics Committee of [redacted] and can be requested if necessary by the researchers. The data was not widely published because it was the privacy of the research participants.

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The Effect of Probiotic Supplementation on The Degree of Depression, Anxiety, and Stress in Chemotherapy Patients: a Pilot Trial

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ABSTRACT

Background: Psychological disorders, including depression, anxiety, and stress, are prevalent among cancer patients undergoing chemotherapy. Probiotics ha~~s~~ve~~s~~ thus been investigated as a potential supplement~~ation~~ to modulate the gut-~~–~~brain axis and improve psychological symptoms through mechanisms such as serotonin regulation~~s~~. However, ~~the~~ study~~y~~ies that specifically examine~~d~~ the effects of probiotics on psychological symptoms in chemotherapy patients are~~is~~ ~~still~~ scarce~~limited~~.

Methods: This randomis~~z~~ed, double-blinded, placebo-controlled pilot trial was conducted at the outpatient clinic of [redacted], in 2023. Sixty-one cancer patients undergoing chemotherapy were enrolled and randomi~~z~~sed into an intervention (~~n~~*n* = 30) and control (~~n~~*n* = 31) group~~s~~. The intervention group~~s~~ received probiotics (*Lactobacillus rhamnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) twice daily for eight~~8~~ weeks. The primary outcomes were changes in depression, anxiety, and stress levels measured by the Depression, ~~-~~Anxiety, and ~~-~~Stress Scale-~~-~~42 (DASS-42). The s~~S~~econdary outcome~~s~~ was ~~included~~ serum serotonin levels.

Results: The intervention group showed a significant decrease~~reduction~~ in total DASS-42 scores (~~p~~*p* = 0.001) after ~~8~~eight weeks, indicating an overall reduction~~decrease~~ in psychological distress. However, changes in the scores of the ~~individual~~ subscales ~~for~~of the DASS-42 were not statistically significant (~~p~~*p* > 0.05). Finally, ~~S~~serum serotonin levels increased in the intervention group, but the change was not statistically significant (~~p~~*p* = 0.382).

Conclusion: While probiotic supplementation significantly reduce~~d~~s overall psychological symptoms, its impact on specific symptoms and serotonin levels ~~was~~is limited. Future research on probiotic supplementation should thus explore longer intervention periods~~,~~ and larger sample sizes, and they should control for external factors more rigorously, to gain a better understanding of the therapeutic impact of probiotics ~~i~~on cancer patients undergoing chemotherapy.

Keywords: probiotics, gut-~~–~~brain axis, psycholog~~y~~ical~~l~~, serotonin, chemotherapy

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1. Introduction

Psychological disorders are becoming increasingly prevalent among patients diagnosed with cancer, with conditions such as delirium, depression, adjustment disorders, anxiety, sexual dysfunction, and sleep disorders affecting 30%–40% of this population. The incidence of psychiatric disorders is even higher among those atin advanced cancer stages of cancer. In Indonesia, 34.4% of cancer patients suffer from depression, with the prevalence increasing rising as the severity and duration of the disease increase. Additionally, a study in one Indonesian hospital reported that 23% of patients undergoing chemotherapy experienced depression, while 40% suffered from anxiety. Despite the significant impact of these conditions on patients' quality of life, psychiatric disorders in cancer patients are often underdiagnosed and inadequately treated, leading to further deterioration in their overall well-being (Mastan et al., 2024; Ostovar et al., 2022; Setiyarini et al., n.d.).

Chemotherapy has been shown to disrupt the balance of gut microbiota, a condition known as gut dysbiosis (Deleemans et al., 2019). This disruption can lead to a reduction in the diversity and number of commensal bacteria, which, in turn, can negatively affect mood and cognitive function (Deleemans et al., 2019; Maddern et al., 2023). Research has demonstrated that changes in microbiota composition can influence the development of the psychological symptoms of conditions including, such as depression and anxiety (Deleemans et al., 2019; Maddern et al., 2023). Animal studies have further demonstrated that gut microbiota plays a critical role in regulating the pathway associated with depression (Deleemans et al., 2019), suggesting that highlighting the potential of gut microbiota may be a promisingas a therapeutic target for psychological disorders amongin cancer patients.

SinceGiven that psychological disorders are linked to low serotonin levels—a condition that can be exacerbated by gut dysbiosis—targeting the gut microbiota through oral probiotics presents a promising therapeutic approach (Merkouris et al., 2024; Zhang et al., 2023). Probiotics have traditionally been used to mitigate the gastrointestinal side effects of chemotherapy, such as nausea and vomiting (Vivarelli et al., 2019). Recent studies have suggested that probiotics may additionally improve psychological well-being by influencing neurotransmitter pathways, including serotonin pathways, which areis closely associated with mood regulation (Merkouris et al., 2024; Zhang et al., 2023). Specifically, probiotics containing such as *Lactobacillus rhamnosus* and *Lactobacillus helveticus* have been shown in animal

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~~studies~~ to reduce symptoms of depression, enhance cognitive function, and balance key neurochemicals, including serotonin, epinephrine, and brain-derived neurotrophic factor (BDNF), in animal studies (Deleemans et al., 2019; Ye et al., 2022).

Despite these promising findings, existing research has predominantly focused on the effects of probiotics on the physical side~~-~~effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions~~symptoms~~ in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) on psychological disorders in a human sample has never been studied ~~to influence psychological disorder in human trial~~. We ~~Therefore, this study~~ aim~~s~~ to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using~~with~~ serum serotonin levels as a potential biomarker for these effects.

2. Method~~s~~

This study was a randomi~~s~~z~~z~~ed, double-blinded, placebo-controlled pilot trial conducted in the~~at~~ o~~O~~utpatient ~~C~~clinic of [redacted] in 2023. The trial was designed to assess the effect of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using~~also~~ serum serotonin levels as a biomarker.

2.1 Participants

Participants included cancer patients who were undergoing chemotherapy in Dr~~.~~ Kariadi Hospital. The only ~~I~~inclusion criteri~~a~~on was that patients had to be ~~required patients~~ aged between 18~~-~~ and 76 years. Exclusion criteria were patients who smoked~~ing~~ or had used antibiotics~~ use~~ during the ~~within one~~ week prior to our ~~before the~~ intervention. Notably,

~~T~~the optimal abstinence period for antibiotics before participating in a ~~entering~~ study assessing probiotics and gut microbiota can vary depending on the type of antibiotic used and the patient's metabolism. Therefore, the abstinence period is usually calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days (Armstrong, 2020). Hence, ~~Thus,~~ a one-week abstinence period preceding ~~before~~ this study ~~entry~~seemed ~~was~~ reasonable to minimi~~z~~se the potential impact of residual antibiotics on gut microbiota and the study outcomes.

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2.2 Sample Size

The sample size was determined using the minimum sample for a ~~of~~ pilot clinical trial (Julious, 2005). A total of 61 patients were enrolled and randomi~~z~~sed into the intervention (~~n~~*n* = 30) and control (*n* ~~n~~= 31) groups, with an allocation ratio~~n~~ of 1:1.

2.3 Randomi~~z~~sation and Blinding

Patients were randomly allocated to either the intervention or control group using block randomi~~z~~sation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was~~will be~~ stored in a sealed envelope and was~~will~~ only ~~be~~ opened after the study was~~is~~ completed. The treatment code was~~is~~ also included ~~inserted~~ i~~nto~~ the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic~~s~~ and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. ~~Probiotics and placebo were given in the form of capsules with the same color, size, and shapes.~~ The placebo capsules were manufactured by the pharmaceutical laboratory of [redacted]. The placebo capsule~~s~~ contained the same additional substances as the probiotic capsule, namely ~~namely~~ maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments;~~.~~ ~~O~~only the pharmacist ~~who~~ knew the group assignments.

2.4 ~~———~~Intervention

The treatment was~~ill be~~ given to the patient upon their arrival~~according to the arrival of the research subject~~. The intervention group received probiotics (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52 at a dose of 2 x 10^9 CFU) twice a day for ~~8~~eight weeks.

2.5 Outcomes

The primary outcome was a~~the~~ change in depression, anxiety, and stress levels, measured using the Depression, ~~-~~Anxiety, and ~~-~~Stress Scale~~-~~ 42 (DASS-42) at baseline and after the ~~8~~eight-week ~~weeks of~~ intervention. The ~~S~~secondary outcome was ~~included~~a change~~s~~ in serum serotonin levels after the eight~~for 8~~ weeks, measure~~s~~d using ~~by~~ an enzyme-linked immunosorbent assay (ELISA).

2.6 Research Instruments

To assess the primary outcomes, the following ~~instrumen~~instrument was~~ts were~~ used:

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1) ~~Depression Anxiety Stress Scale 42 (~~DASS-42~~)~~

The DASS-42 is a 42-item questionnaire used to measure the severity of depression, anxiety, and stress in participants. The questionnaire consists of 42 assessment statements (T~~t~~able 1) to assess depression (14 statements), anxiety (14 statements), and stress (14 statements). Participants were asked to score each statement as ~~with~~ 0 = *never*, 1 = *sometimes*, 2 = *often*, or ~~and~~ 3 = *very often*. Subscale scores were then summed to determine the depression, anxiety, and stress scale scores (T~~t~~able 2).

Meanwhile, to assess the secondary outcome, the following instrument was~~ were~~ used:

2) Serotonin measurement

Serum serotonin levels were measured using an ~~enzyme-linked immunosorbent assay (~~ELISA~~)~~, following the manufacturer's protocol. Blood samples were collected at baseline and after the intervention, and results were read using a microplate reader (EL x 800).

2.7 Statistical Methods

Data were analy~~z~~sed using intention-to-treat principles. Continuous variables (age, DASS-42 score, serum serotonin level) were summari~~z~~sed using mean ± standard deviation, while categorical variables (gender, marital status, educational level, ~~i~~occupation, history of psychiatric illness, history of psychiatric treatment, and duration of cancer diagnosis) were presented as frequencies and percentages. The Shapiro~~aphiro-~~ Wilk test was used to assess the normality of data distribution. Between-group comparisons were conducted using the Mann-Whitney U test for continuous variables and the c~~C~~hi-square test for categorical variables. Within-group comparisons were conducted~~analyzed~~ using the Wilcoxon signed rank test. A *p-p=*value < 0.05 was considered statistically significant.

2.8 Ethics

The study was registered at the Indonesian Clinical Research Registry (INA-CRR) with registration number 042024030706474KRGNHZ, and it was approved by the Health Research Ethics Committee of [redacted] (No. 1496/EC/KEPK-RSDK/2023). All

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participants provided written informed consent prior to participation. The trial was conducted according to the principles of the Declaration of Helsinki.

3. Results

3.1 Sample Ceharacteristics

This study includedinvolved 61 cancer patients who met the inclusion and exclusion criteria. The study sample was divided into 2two groups byvia randomiszation, namely anthe intervention group and athe control group. A total of two2 research subjects from the intervention group died during the trial, 4four subjects werecare hospitalizsed during the trial, 3three subjects did not receive their come to control for treatment, and 8eight subjects wereare lostsˌ to follow-up, so the intervention group comprisedconsisted of 13 subjects. Meanwhile, 2two subjects from the control group died during the trial, one 1 subject dropped out from taking medicine, 3three subjects were hospitalizsed during the trial, 4four subjects did not receive theircome to control for treatment, and 15 subjects wereare lostsˌ to follow-up, so the control group also comprisedconsisted of 13 subjects (Figure 1).

Table 3 shows the characteristics of the study sample forin each intervention group and control group. In this study sample, the mean ages in the intervention and control groups wereas 54,.46 and 49,.08 years, respectively. The number of male and female patients was approximately equal in the intervention and control groups. Most of the study sample waseare married (84,.6%), had high school as their highest level of educationhad the highest education level of high school (30.7%), and worked (65%). All samples had no history of psychiatric treatment or previous psychiatric diagnosciˌs. There were also no significant differences in their age, gender, marital status, educational level, employment status, and duration of cancer, either diagnosis.

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3.2 The Effect of Probiotics on Depression, Stress, and Anxiety

The results showed an insignificant decrease in depression ~~scores~~ ($~~p~~p$ = 0.317), anxiety ($~~p~~p$ = 0.914), and stress ($~~p~~p$ = 0.581) scores, ~~,~~ while there was a ~~result show~~ significant decrease in total DASS-42 scores ($~~p~~p$ = 0.001), in the intervention group after receiving~~administering~~ probiotics for ~~8~~eight weeks (Table 4). When ~~While from the~~ comparing~~son of~~ the control group with the intervention group, there were insignificant differences in~~between the scores~~ depression, anxiety, and stress scores, but total~~the scores of total~~ DASS-42 scores ~~in the interventio~~significantly differed between the two groups~~n group and the control group had significant difference after administering probiotics for 8 weeks~~ ($~~p~~p$ = 0.048; ~~) (~~ Table 4).

3.3 The Effect of Probiotics on Serotonin

We found ~~In this study, there was~~ an increase in serotonin levels in the intervention group ~~with probiotic administration~~, but it was not statistically significant ($~~p~~p$ = 0.382; ~~). (~~Table 5).

4. Discussion

Cancer patients undergoing chemotherapy frequently experience significant psychological distress, including depression, anxiety, and stress, which adversely affects their quality of life (Ostovar et al., 2022). Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence the psychological symptoms of~~, such as~~ depression, anxiety, and stress via ~~through~~ the gut–brain axis (Sabit et al., 2023). A systematic review also reported the beneficial effects of probiotic supplementation measured using the~~on~~ Hamilton Depression Rating Scale ~~(HAMD)~~ in patients with psychiatric disorders (Amirani et al., 2020). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris et al., 2024; Potter et al., 2023; Ye et al., 2022; Zhang et al., 2023).

In our ~~this current~~ study, the administration of probiotics over eight ~~an 8~~ weeks~~ period~~ caused a significant decrease in ~~the~~ total DASS-42 scores in the intervention group ($~~p~~p$ = 0.001), indicating an overall reduction in psychological distress. However, the decreases~~reductions of~~ in the scores for each sub-scale~~s~~ (depression, anxiety, and stress) were not statistically significant ($~~p~~p$ > 0.005). These findings suggest that~~,~~ despite the beneficial effect of probiotics on overall

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psychological symptoms, as measured using ~~by the~~ total ~~of~~ DASS-42 scores, the impact of probiotics on specific symptoms (depression, anxiety, and stress) may be more limited or may require a longer intervention ~~duration on intervention~~ to become significant.

Chemotherapy, radiotherapy, and immunotherapy have toxic~~ity~~ effects that can lead to changes in gut microbiota, a reduction in~~of~~ gut commensal bacteria, and inflammation of the gastrointestinal tract. Research has also found a bidirectional relationship between the digestive system and the nervous system (gut-~~–~~brain axis; ~~)(~~Deleemans et al., 2019; Ichim et al., 2018; Maddern et al., 2023; Vivarelli et al., 2019). Gut dysbiosis can lead to increased gut permeability, allowing toxins to enter the bloodstream and activating~~cause the activation of~~ pro-inflammatory cytokines (IL-6, II-1b, TNF-a, and C-reactive protein ~~(CRP)~~), while it may cause ~~and~~the hyperactivation of the hypothalamic-~~–~~pituitary axis (HPA). These inflammatory conditions then lead to decreased levels of serotonin (5-HT) and ~~brain-derived neurotrophic factor (~~BDNF~~)~~. Both of these can, in turn, ~~-~~cause psychological and cognitive changes such as anxiety and depression, fatigue, memory impairment, and decision-making impairment~~ed decision-making~~ (Lu et al., 2022; Maddern et al., 2023; Merkouris et al., 2024; Sabit et al., 2023).

The mechanism of psychological disturbance discussed in our~~this~~ study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant (*p*p = 0.0382). ~~Interestingly, T~~the control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, ~~T~~there are certain external factors that may have influenced~~s~~ these results.

One such factor is the potential consumption of ondansetron ~~by patients~~. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels, potentially explaining the more pronounced changes observed in the control group.

In addition, a diet~~ary intake~~ that includes amino acids such as tryptophan, a precursor of serotonin, can~~ould have~~ contribute~~d~~ to variations in serotonin levels (Jenkins et al., 2016; Mohajeri et al., 2015) since~~.~~ ~~T~~tryptophan-rich foods can increase serotonin synthesis. However,

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dietary habits were not controlled for in this study, even thoughthus, the differences in dietsary intake among participants might have affected the results. Future studies should thus aim to control for the medications and dietsary intake of their participants more rigorously.

AnThe increase in serotonin is one of several different mechanisms involved in the improvement of depression, anxiety, and stress symptoms from probiotic use. In addition to serotonin production, probiotics may reduce symptoms of depression and anxiety bythrough several different mechanisms, including decreasing stress-induced HPA responses, decreasinglowering cortisol levels, increasing neurotransmitter synthesis (GABA, dopamine, noradrenaline, melatonin, histamine, and acetylcholine), stimulating the production of gut neuropeptides ([glucagons like peptide-1 (GLP-1) and tyrosine (PYY)]), improving the gut barrier, increasing BDNF production, and decreasing the release of pro-inflammatory cytokines, and increasing anti-inflammatory cytokines (Lu et al., 2022; Sabit et al., 2023; Zhang et al., 2023).

Overall, the Ggut–brain axis and serotonin production are influenced by numerousa complex of factors beyond just probiotic supplementation, includingfor example stress levels, diet, and physical activity (Lou et al., 2023; Madison and Kiecolt-Glaser, 2019; Mohajeri et al., 2015). The influence of these variables might have differed between the control and intervention groups, contributing to the outcomes seen in our study.

4.1 The study lLimitations

This study hased several limitations. Specifically, we used , such as the use of serum serotonin as a biomarker. While the direct impact of CNS serotonin on the pathophysiology of depression, anxiety, and stress is already well-recognizsed, to have direct implication in the pathophysiology of depression, anxiety, and stress, the direct measurement of CNS serotonin is invasive and not feasible in a clinical trial setting. Therefore, us, in this study, we used serum serotonin levels as a biomarker to were measures as a biomarker to assess the potential impact of probiotic supplementation on serotonin levels. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may do not directly accurately reflect CNS serotonin concentration. AlThough this wasis a limitation ofin our the methodology, it is considered a feasible and ethical of non-invasive sampling method forin human subjects. Moreover, serum serotonin remains a valuable biomarker in studies exploring the gut–brain axis

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and the systemic effects of probiotic supplementation (Merkouris et al., 2024). This is because ~~P~~previous studies have reported that gut microbiota can modulate systemic serotonin levels, which, in turn, may influence CNS function through the gut~~-~~–brain axis (Jenkins et al., 2016; Potter et al., 2023; Yano et al., 2015).

Another limitation of this study was~~is~~ the ~~relatively~~ brevity ~~short duration~~ of the intervention. Eight weeks~~The 8-week period~~ may not have been sufficient to observe significant changes in ~~specific~~ the psychological conditions~~symptoms~~ of~~, such as~~ depression, anxiety, and stress. Studies have suggested that ~~the~~ gut microbiota exist in ~~is~~ a complex and dynamic ecosystem that may require longer ~~periods of~~ probiotic interventions to undergo~~achieve~~ significant alterations (Ng et al., 2023). Another study also reported the~~the~~ beneficial effects of probiotic supplementation on~~were seen on~~ symptoms of depression ~~after probiotic supplementation for~~after 12 weeks among~~to~~ patients under methadone maintenance treatment programmes ~~(MMTP)~~ (Molavi et al., 2022).

Additionally, we~~the study~~ did not control for physical activity, dietary habits, symptomatic medications, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients ~~of the patients~~.

Based on these limitations, ~~considerations previously explained,~~ future research should explore the effects of longer-term probiotic supplementation and control for external factors more rigorously, perhaps through more detailed dietary assessments, physical activity logs, and the closer monitoring of medication use.

5. Conclusions

We ~~The study~~ found that an ~~8~~eight-week probiotic supplementation regimen significantly decrease~~d~~s overall psychological symptoms, as measured by ~~the~~ total DASS-42 scores. However, we found~~there was~~ no statistically significant changes in the depression, anxiety, and stress ~~specific~~ subscale scores of participants~~, such as depression, anxiety, and stress~~. In addition, even though the serum serotonin levels in the intervention group increased, this alteration was not statistically significant. Hence, ~~In conclusion,~~ the results of this pilot study show the promise of probiotic supplementation for psychological symptoms among cancer patients ~~are quite promising~~ and should be extended~~continued~~ with longer intervention periods, larger sample

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sizes, and stricter inclusion and exclusion criteria, in addition to controlling for other influencing factors.

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Second revision submitted (20-09-2024)

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Manuscript Title: Probiotic Supplementation Effects on Depression, Anxiety, and Stress in Cancer Patients on Chemotherapy via Serotonin Biomarker Analysis: A Pilot Trial
Journal: Mental Health & Prevention

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Revisions and Amends

Points-by-Points Revision

Manuscript: Fitrikasari et al. “Probiotic Supplementation Effects on Depression, Anxiety, and Stress in Cancer Patients on Chemotherapy via Serotonin Biomarker Analysis: A Pilot Trial”

Dear Editor in Chief and reviewers,

We really appreciate the constructive feedback you have provided in the first-round review of our manuscript. We attempted to address all suggestions and comments meticulously. As suggested, we have formatted the revision so that the changes can be tracked. The details of the revision are as follow:

REVIEWER 1			
No	Review	Revision	Page
Title			
1	The study title should indicate that this is a pilot trial.	Thank you for the suggestion. We have revised the title by adding the word “pilot trial” The Effect of Probiotic Supplementation on the Degree of Depression, Anxiety, and Stress in Cancer Patients on Chemotherapy via Serotonin Biomarker Analysis: A Pilot Trial	Title page
Introduction			
1	Please note that scientific names such as "Lactobacillus rhomnosus" should be italicized as per convention.	Thank you very much for this detailed comment. We have revised all the scientific names according to the rules. The intervention group received probiotics (*Lactobacillus rhamnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) twice daily for eight weeks. Specifically, probiotics containing *Lactobacillus rhamnosus* and *Lactobacillus helveticus* have been shown to reduce symptoms of depression, enhance cognitive function, and balance key neurochemicals, including serotonin, epinephrine, and brain-derived neurotrophic factor (BDNF), in animal studies (Deleemans et al., 2019; Ye et al., 2022). In addition, the influence of the combination of probiotics used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) on psychological disorders in a human sample has never been studied.	 Page 1, line 11-12 Page 2, line 55-56 Page 3, line 63

		The treatment was given to the patient upon their arrival. The intervention group received probiotics (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52 at a dose of 2 x 10^9 CFU) twice a day for eight weeks.	Page 4, line 100
2	The manuscript is in general need of language editing. Suggest a close proofreading ideally by a native English speaker. For example, "Previous studies have shown that cancer patients experience depression and anxiety, which usually appear in the early phase and during treatment" could be shortened to: "Cancer patients commonly experience depression and anxiety during treatment." "8 subjects are loss to follow up" is also incorrect, it should be "lost to follow up" and not "loss".	Thank you very much for your feedback on this. We have completed the proofreading process by a professional agent (Cambridge proofreading). The whole sections have been proofread, and we hope that the manuscript is clearer.	
Methods			
1	In the methods section, please ensure that the trial is reported according to the CONSORT guidelines (citation: ncbi.nlm.nih.gov/pmc/articles/PMC2857832).	We revised the methods section based on the CONSORT guidelines in the link you have provided. It consists of trial design, participants, interventions, outcomes, sample size, randomization, implementation, blinding, and statistical methods. The revised version is provided in the methods section of the manuscript.	Page 3-5
2	The rationale for measuring serum serotonin levels should be at least briefly explained, and with appropriate supporting references. While serum serotonin levels can be indicative of systemic changes and are easily accessible for sampling, they may not accurately reflect the CNS serotonin levels. Serum serotonin is largely stored in platelets and can be influenced by factors unrelated to CNS serotonin activity.	Thank you for your comment on this. We have added the explanation why we measured serum serotonin as a biomarker in the limitation part. Although we wrote it as one of the limitations of our study, we believe that it is the most feasible and non-invasive sampling method to predict the CNS serotonin level. While the direct impact of CNS serotonin on the pathophysiology of depression, anxiety, and stress is well-recognised, the direct measurement of CNS serotonin is invasive and not feasible in a clinical trial setting. Therefore, in this study, we used serum serotonin levels as a biomarker to measure the potential impact of probiotic supplementation on serotonin levels. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may not accurately reflect CNS serotonin concentration. Although this was a limitation of our methodology, it is considered a feasible and ethical non-invasive sampling method for human subjects. Moreover, serum serotonin remains a valuable biomarker in studies exploring the gut–brain axis and the systemic effects of probiotic supplementation (Merkouris et al., 2024). This is because previous studies have reported that gut microbiota can modulate systemic serotonin levels, which, in turn, may influence CNS	Page 8, line 227-239

		function through the gut–brain axis (Jenkins et al., 2016; Potter et al., 2023; Yano et al., 2015).	
3	How was the sample size determined? At least some elaboration is necessary.	We added the explanation on our reference to determine the sample size on the methods section (https://doi.org/10.1002/pst.185). The sample size was determined using the minimum sample for a pilot clinical trial (Julious, 2005). A total of 61 patients were enrolled and randomized into the intervention (n = 30) and control (n = 31) groups, with an allocation ratio of 1:1.	Page 3, line 83-85
4	Several aspects of the design need to be explained in greater detail. Please further explain the process of ensuring adherence to the probiotic regimen, as well as the exact procedures for randomization, allocation concealment, and blinding, to ensure reproducibility and replicability.	Thank you for the suggestion. We have revised the subsection of randomization and blinding to give clearer study procedures and ensure that it can be replicated in other study. Patients were randomly allocated to either the intervention or control group using block randomization, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of Medical Faculty, Universitas Diponegoro. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.	Page 4, line 87-97
5	The use of parametric tests (e.g., t-tests, ANOVA) on DASS scores can be problematic due to the ordinal nature of the data. Non-parametric tests (e.g., Mann-Whitney U, Wilcoxon signed-rank test) are more appropriate as they do not assume equal intervals.	Thank you very much for this feedback. We have revised the data analysis section in more details. We interpreted DASS 42-scores as continuous data because we reported the overall and each subscale mean scores. We did not categorized the subscale score, for example depression into mild, moderate, and severe. Thus, we still used t-tests if the data was normally distributed and used non-parametric test if the data was not normally distributed. Continuous variables (age, DASS-42 score, and serum serotonin level) were summarised using mean ± standard deviation, while categorical (nominal and ordinal) variables (gender, marital status, highest educational, jobs, history of psychiatric illness, history of psychiatric treatment, and duration of cancer diagnosis) were presented as frequencies and percentages.	Page 5, line 122-134

		For nominal variables (gender, marital status, and jobs), between group comparisons were conducted using chi-square test. Meanwhile, Mann-Whitney test was used for ordinal variables (highest education and duration since cancer diagnosis). For continuous variables, Shapiro–Wilk test was used to assess the normality of data distribution. Between-group comparisons were conducted using independent t-test if the data was normally distributed and Mann–Whitney U test if the data was not normally distributed. Within-group comparisons were conducted using paired t-test if the data was normally distributed and using Wilcoxon signed rank test if the data was not normally distributed. A p-value < 0.05 was considered statistically significant	
Results			
1	Please correct the stylistic and typo errors. It should be "54.46" and "49.08" rather than "54,46" and "49,08". Similarly, "0,710" should be "0.710".	Thank you very much for this detailed comment. We have revised all the number style error according to your suggestions. It is provided in the tables.	
2	There is no point reporting age with two decimal places. It is also more useful to report age using median and IQR.	Thank you again for this detailed suggestion. We have revised the mean age without decimal. It is provided in table 3.	
3	In Table 3, please change "Divorce" to "Divorced".	Thank you for this detailed comment. We have revised the word in table 3.	
Discussion			
1	Regarding probiotics, the shift in the gut microbiota may be transient and temporary (citation: pubmed.ncbi.nlm.nih.gov/36986088) as several treatment trials for probiotics have failed to find significant alterations in gut microbiome; individuals may require longer duration of treatment to have therapeutic effects. This should be discussed.	Thank you very much for your feedback. We added the intervention duration as one of the limitations in our study. It can be a recommendation for further study to conduct longer intervention. Another limitation of this study was the brevity of the intervention. Eight weeks may not have been sufficient to observe significant changes in the psychological conditions of depression, anxiety, and stress. Studies have suggested that gut microbiota exist in a complex and dynamic ecosystem that may require longer probiotic interventions to undergo significant alterations (Ng et al., 2023). Another study also reported the beneficial effects of probiotic supplementation on symptoms of depression after 12 weeks among patients under methadone maintenance treatment programmes (Molavi et al., 2022).	Page 9, line 240-246
2	The discussion of results is also overly optimistic without sufficient consideration of the limitations.	Thank you very much for the feedback. We added several limitations of our study that we hope can be considered for further study in the same context. This study had several limitations. Specifically, we used serum serotonin as a biomarker. While the direct impact of central nervous system (CNS) serotonin on the pathophysiology of depression, anxiety, and stress is well-recognised, the direct measurement of CNS serotonin is invasive and not feasible in a	Page 8-9, line 227-253

		clinical trial setting. Therefore, in this study, we used serum serotonin levels as a biomarker to measure the potential impact of probiotic supplementation on serotonin levels. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may not accurately reflect CNS serotonin concentration. Although this was a limitation of our methodology, it is considered a feasible and ethical non-invasive sampling method for human subjects. Moreover, serum serotonin remains a valuable biomarker in studies exploring the gut–brain axis and the systemic effects of probiotic supplementation (Merkouris et al., 2024). This is because previous studies have reported that gut microbiota can modulate systemic serotonin levels, which, in turn, may influence CNS function through the gut–brain axis (Jenkins et al., 2016; Potter et al., 2023; Yano et al., 2015). Another limitation of this study was the brevity of the intervention. Eight weeks may not have been sufficient to observe significant changes in the psychological conditions of depression, anxiety, and stress. Studies have suggested that gut microbiota exist in a complex and dynamic ecosystem that may require longer probiotic interventions to undergo significant alterations (Ng et al., 2023). Another study also reported the beneficial effects of probiotic supplementation on symptoms of depression after 12 weeks among patients under methadone maintenance treatment programmes (Molavi et al., 2022). Additionally, we did not control for physical activity, dietary habits, symptomatic medications, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Based on these limitations, future research should explore the effects of longer-term probiotic supplementation on the psychological symptoms of cancer patients undergoing chemotherapy and control for external factors more rigorously, perhaps through more detailed dietary assessments, physical activity logs, and the closer monitoring of medication use.	

REVIEWER 2			
No	Review	Revision	Page
Abstract			
1	In the abstract, conclusion should be edited. As it has been written with too much assertion. The conclusion should be limited to the results of the study and not exceed any more. It should only implicate the extraction of your own results.	Thank you very much for your feedback. We have revised the conclusion part of the abstract to implicate the extraction of study's result only. Conclusion: An eight-week probiotic supplementation significantly reduced overall psychological symptoms, as shown by total DASS-42 scores, but did	Page 1, line 20-23

		not lead to significant changes in depression, anxiety, or stress subscale scores. Serum serotonin levels also increased in the intervention group, though not significantly.	
Introduction			
1	The last paragraph of introduction is not well-written. The authors should imply to the necessity of their work much better.	Thank you very much for your suggestion. We have revised the section to explain the novelty of our study. We hope that it is clearer why we chose to investigate the effect of probiotic supplementation on psychological symptoms of caner patients undergoing chemotherapy. Despite these promising findings, existing researches have predominantly focused on the effects of probiotics on the physical side-effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (Lactobacillus rhomnosus Rosell-11 and Lactobacillus helveticus Rosell-52) on psychological disorders in a human sample has never been studied. We aim to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using serum serotonin levels as a potential biomarker for these effects.	Page 3, line 59-66
Methods			
1	The manuscript needs English polishing and appropriate reporting according to CONSORT statement.	Thank you very much for your feedback on this. We have completed the proofreading process by a professional agent (Cambridge proofreading). The whole sections have been proofread, and we hope that the manuscript is clearer. We revised the methods section based on the CONSORT statement. It consists of trial design, participants, interventions, outcomes, sample size, randomization, implementation, blinding, and statistical methods. The revised version is provided in the methods section of the manuscript.	
2	It is exactly known whether the study design is interventional or randomized controlled trial. If it was carried out randomized manner, then why consecutive sampling method was applied? If it is an RCT then it should be mentioned everywhere especially in the title of the study. In part 2.3.1. It is implied to an RCT design, but in the above paragraph, other method i.e. consecutive sampling is mentioned.	Thank you for the correction. We have revised the sampling method by doing randomization and blinding. The exact procedures for randomization, allocation concealment, and blinding have also been explained. We hope the sampling method is clearer after the revision. Patients were randomly allocated to either the intervention or control group using block randomisation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the	Page 4, line 87-97

		block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of Medical Faculty, Universitas Diponegoro. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.	
3	What were blocks stratified for?	Thank you for your question. We did not use blocks stratified. We randomly allocated the participants to either the intervention or control group using block randomization with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1.	
4	Part 2.3.1. is written with future verbs!!	Thank you for the correction. We have revised the verbs into past tense. Patients were randomly allocated to either the intervention or control group using block randomisation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of Medical Faculty, Universitas Diponegoro. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.	Page 4, line 87-97
5	Was one week of antibiotic abstinence enough to allow someone to enter the study?	Thank you for the question. The optimal abstinence period for antibiotics before entering study assessing probiotic and gut microbiota can vary depending on the type of antibiotic and patient's metabolism. Therefore, the abstinence period is calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days. Thus, a one-week abstinence period before study entry was reasonable to minimize the potential impact of residual antibiotics on gut microbiota and study outcomes.	Page 3, line 76-81
6	Exclusion criteria seem not to be complete.	Thank you for the comment. The exclusion criteria have already completed. They were smoking or antibiotic use within one week before the intervention.	

7	Figure one as the flowchart of the study is not acceptable. Figure 2 is enough.	Thank you for your suggestion. We have deleted the figure one.	
8	Whole verbs used in the study should be written in the simple past tense.	Thank you very much for your feedback on this. We have completed the proofreading process by a professional agent (Cambridge proofreading). The whole sections have been proofread, and we hope that the grammas is correct.	
9	The method of analysis of normally and abnormally distributed data is not separately written. If it is an RCT, then, ANCOVA test should have been conducted.	Thank you very much for your feedback. We added the explanation of analysis data and separated the normally and abnormally distributed data. However, with the data we have, different age categories have the potential to be included as confounding factors, but the ANCOVA test could not be performed properly due to the limited number of subjects.	
Results			
1	In Table 3, plz merge numbers under 5 and avoid presenting the results in this way	Thank you for your suggestion. We have revised table 3 and put maximum two decimals.	
2	**KT should be plus placebo	Thank you for your correction. We have revised it into KT=chemotherapy+placebo	
3	Tables 4 to 7 should be merged and presented in one Table. Further, as I stated above, ANCOVA test should be used for inter-group comparisons.	Thank you for your suggestion. We have merged table 4 to 7 into one table (table 4). However, as we said before, we could not perform ANCOVA test due to the limited number of subjects.	
Discussion			
1	According to the authors, "In this study, there was an increase in serotonin levels in the intervention group with probiotic administration, but it was not statically significant (p=0,382). (Table 8)". However, the authors have not paid attention to more noticeable changes in the control group than the intervention group. In most may be all parts, the changes are more observable in the control group!!	Thank you for your feedback. We acknowledge that the changes in serotonin levels were more observable in the control group than the intervention group. It may be because of certain external factors, such as consumption of antiemetic medicine (ondansetron) by patients or dietary intake that includes amino acids. More explanation has been elaborated in the discussion part of the manuscript. The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant (p = 0.0382). The control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, there are certain external factors that may have influenced these results. One such factor is the potential consumption of ondansetron. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating	Page 7-8, line 196-212

		serotonin levels, potentially explaining the more pronounced changes observed in the control group. In addition, a diet that includes amino acids such as tryptophan, a precursor of serotonin, can contribute to variations in serotonin levels (Jenkins et al., 2016; Mohajeri et al., 2015) since tryptophan-rich foods can increase serotonin synthesis. However, dietary habits were not controlled for in this study, even though differences in diets among participants might have affected the results. Future studies should thus control for the medications and diets of their participants more rigorously.	
2	The authors have only discussed the mechanisms and nothing is discussed on the results of the study!	Thank you for your feedback. We have revised the discussion part. So, it did not only explain about the mechanism, but also the elaboration of the study's result. Cancer patients undergoing chemotherapy frequently experience significant psychological distress, including depression, anxiety, and stress, which adversely affects their quality of life (Ostovar et al., 2022). Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence the psychological symptoms of depression, anxiety, and stress via the gut–brain axis (Sabit et al., 2023). A systematic review also reported the beneficial effects of probiotic supplementation measured using the Hamilton Depression Rating Scale in patients with psychiatric disorders (Amirani et al., 2020). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris et al., 2024; Potter et al., 2023; Ye et al., 2022; Zhang et al., 2023). In our study, the administration of probiotics over eight weeks caused a significant decrease in total DASS-42 scores in the intervention group ($p = 0.001$), indicating an overall reduction in psychological distress. However, the decreases in the scores for each subscale (depression, anxiety, and stress) were not statistically significant ($p > 0.005$). These findings suggest that despite the beneficial effect of probiotics on overall psychological symptoms, as measured using total DASS-42 scores, the impact of probiotics on specific symptoms (depression, anxiety, and stress) may be more limited or may require a longer intervention to become significant. Chemotherapy, radiotherapy, and immunotherapy have toxic effects that can lead to changes in gut microbiota, a reduction in gut commensal bacteria, and inflammation of the gastrointestinal tract. Research has also found a bidirectional relationship between the digestive system and the nervous system (gut–brain axis; Deleemans et al., 2019; Ichim et al., 2018; Maddern et al., 2023; Vivarelli et al., 2019). Gut dysbiosis can lead to increased gut	Page 6-8, line169-225

		permeability, allowing toxins to enter the bloodstream and activating pro-inflammatory cytokines (IL-6, II-1b, TNF-a, and C-reactive protein), while it may cause the hyperactivation of the hypothalamic–pituitary axis (HPA). These inflammatory conditions then lead to decreased levels of serotonin (5-HT) and BDNF. Both of these can, in turn, cause psychological and cognitive changes such as anxiety and depression, fatigue, memory impairment, and decision-making impairment (Lu et al., 2022; Maddern et al., 2023; Merkouris et al., 2024; Sabit et al., 2023). The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.0382$). The control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, there are certain external factors that may have influenced these results. One such factor is the potential consumption of ondansetron. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels, potentially explaining the more pronounced changes observed in the control group. In addition, a diet that includes amino acids such as tryptophan, a precursor of serotonin, can contribute to variations in serotonin levels (Jenkins et al., 2016; Mohajeri et al., 2015) since tryptophan-rich foods can increase serotonin synthesis. However, dietary habits were not controlled for in this study, even though differences in diets among participants might have affected the results. Future studies should thus control for the medications and diets of their participants more rigorously. An increase in serotonin is one of several mechanisms involved in the improvement of depression, anxiety, and stress symptoms from probiotic use. In addition to serotonin production, probiotics may reduce symptoms of depression and anxiety by decreasing stress-induced HPA responses, decreasing cortisol levels, increasing neurotransmitter synthesis (GABA, dopamine, noradrenaline, melatonin, histamine, and acetylcholine), stimulating the production of gut neuropeptides (glucagons like peptide-1 and tyrosine), improving the gut barrier, increasing BDNF production, decreasing the release of pro-inflammatory cytokines, and increasing anti-inflammatory cytokines (Lu et al., 2022; Sabit et al., 2023; Zhang et al., 2023). Overall, the gut–brain axis and serotonin production are influenced by numerous factors beyond just probiotic supplementation, including stress	

		levels, diet, and physical activity (Lou et al., 2023; Madison and Kiecolt-Glaser, 2019; Mohajeri et al., 2015). The influence of these variables might have differed between the control and intervention groups, contributing to the outcomes seen in our study.	
3	The conclusion is very weak.	Thank you for your feedback. We have revised the conclusion part to summarize the study's result and recommendation for further study. We found that an eight-week probiotic supplementation regimen significantly decreases overall psychological symptoms, as measured by total DASS-42 scores. However, we found no statistically significant changes in the depression, anxiety, and stress subscale scores of participants. In addition, even though the serum serotonin levels in the intervention group increased, this alteration was not statistically significant. Hence, the results of this pilot study show the promise of probiotic supplementation for psychological symptoms among cancer patients and should be extended with longer intervention periods, larger sample sizes, and stricter inclusion and exclusion criteria, in addition to controlling for other influencing factors.	Page 9, line 255-262

REVIEWER 3			
No	Review	Revision	Page
Abstract			
1	The keywords should be taken from the Medical Subject Headings.	Thank you for your suggestion. We have revised the keywords according to the Medical Subject Headings. Keywords: probiotics, gut–brain axis, psychology, serotonin, chemotherapy	Page 1, line 24
Introduction			
1	Please describe more about the novelty of manuscript in the introduction.	Thank you for your suggestion. We have described more about the novelty of our study in the last paragraph of the introduction section. Despite these promising findings, existing researches have predominantly focused on the effects of probiotics on the physical side-effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (Lactobacillus rhomnosus Rosell-11 and Lactobacillus helveticus Rosell-52) on psychological disorders in a human sample has never been studied. We aim to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and	Page 3, line 59-66

		stress in cancer patients undergoing chemotherapy, using serum serotonin levels as a potential biomarker for these effects.	
Methods			
1	What has been the basis for the classification of the groups?	We classified the groups into intervention and control group based on block randomization. We have elaborated it in randomization and blinding subsection. Patients were randomly allocated to either the intervention or control group using block randomisation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of [redacted]. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.	Page 4, line 87-97
2	What is the justification in your choice of Probiotic dose?	Thank you for your question. This study used the probiotic strains *Lactobacillus rhomnosus* Rossel 11 and *Lactobacillus helveticus* Rossel 52, which have generally been shown to reduce serotonin levels and symptoms of depression, either alone or in combination. The specific role of each probiotic, and the optimal dose of probiotics are still not well understood. The combination used in previous trials included Lactobacillus and Bifidobacterium, with study durations varying from 3-24 weeks. Therefore, the probiotic dose was based on previous study. The intervention group received probiotics (Lactobacillus rhomnosus Rosell-11 and Lactobacillus helveticus Rosell-52 at a dose of 2 x 109 CFU) twice a day for eight weeks.	
3	Is there a precise documentation of side effects following international standards? Please provide this information	Thank you for your question. There was no any side effect documented because of probiotic supplementation in this study.	
4	Did not people study smoking and drug use? Do not use hypnotic drugs? Do you think these do not interfere with the study? How are they controlled?	Thank you for your question. The respondents used chemotherapy drugs but not antipsychotic drugs, such as benzodiazepine. It was already mentioned in the characteristics of the patient. One drug that might influence serum serotonin level is ondansetron. We explained it in the discussion part.	

		The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.0382$). The control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, there are certain external factors that may have influenced these results. One such factor is the potential consumption of ondansetron. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels, potentially explaining the more pronounced changes observed in the control group. However, we did not control it. Thus, we mentioned it as one of limitations of this study. Additionally, we did not control for physical activity, dietary habits, symptomatic medications, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients.	Page 7, line 196-206 Page 9, line 247-249
5	Blindness protocol and labeling methods should be described more comprehensively.	Thank you for your suggestion. We have described the blindness protocol and labeling more comprehensive. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration.	Page 4, line 89-92
6	No information about the physical activity records.	Thank you for your comment. Yes, we did not record the physical activities of the respondents. Thus, we mentioned it as one of the study limitations. Additionally, we did not control for physical activity, dietary habits, symptomatic medications, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients.	Page 9, line 247-249
7	What was the composition of the placebo?	Thank you for your question. The placebo capsules contain the same additional substance as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results.	

8	Please describe the statistical analyses in more detail. For example, did you adjust the outcome variable measured in the follow-up measurements for the baseline value of the outcome (according to the equation $Yt = \beta0 + \beta1* X + \beta1*Yt0$, where Yt = the outcome measured in the two follow-up measurements, X = treatment variable, β1 = overall treatment effect, and Yt0 = outcome variable measured in the baseline measurement)? Please indicate the parameters you have adjusted for in your linear regression analysis. It is well acceptable that an appropriate significance level α, such as 0.05, is pre-specified to guarantee the probability of incorrectly rejecting a single test of null hypothesis (H0) no larger than α. However, there are many situations where more than one or even a large number of hypotheses are simultaneously tested, which is referred to as multiple comparisons. Because you are testing many different hypothesis simultaneously ("multiple comparisons"), proper adjustment of statistical inference is required.	Thank you for your feedback. With the data we have, different age categories have the potential to be included as confounding factors, but the ANCOVA test cannot be performed properly due to the limited number of subjects.	
9	Identify the primary and secondary outcomes.	Thank you for your comment. The primary outcome was the change in depression, anxiety, and stress levels, measured using the Depression-Anxiety-Stress Scale-42 (DASS-42) at baseline and after 8 weeks of intervention. Secondary outcome included changes in serum serotonin levels for 8 weeks, measures by enzyme-linked immunoassay (ELISA).	
Results			
1	The table result is unclear. Please state the changes between the two groups in the variables examined, also β (95% CI).	Thank you for your comment. We have revised and merged the table from table 4 to 7 into table 8. We hope it is clearer.	
Discussion			
1	In the discussion, refer to the new article published in this field." The Effects of Probiotic Supplementation on Opioid-Related Disorder in Patients under Methadone Maintenance Treatment Programs". And https://doi.org/10.1016/j.ctim.2020.102361	Thank you for your suggestion. Those articles have been cited in the manuscript. A systematic review also reported the beneficial effects of probiotic supplementation measured using the Hamilton Depression Rating Scale in patients with psychiatric disorders (Amirani et al., 2020).	Page 6, line 173-175 Page 9, line 244-246

		Another study also reported the beneficial effects of probiotic supplementation on symptoms of depression after 12 weeks among patients under methadone maintenance treatment programmes (Molavi et al., 2022).	

Revised version

ABSTRACT

Background: Psychological disorders, including depression, anxiety, and stress, are prevalent among cancer patients undergoing chemotherapy. Probiotics have thus been investigated as a potential supplement to modulate the gut–brain axis and improve psychological symptoms through mechanisms such as serotonin regulation. However, studies that specifically examine the effects of probiotics on psychological symptoms in chemotherapy patients are scarce.

Methods: This randomised, double-blinded, placebo-controlled pilot trial was conducted at the outpatient clinic of [redacted], in 2023. Sixty-one cancer patients undergoing chemotherapy were enrolled and randomised into an intervention (n = 30) and control (n = 31) group. The intervention group received probiotics (*Lactobacillus rhamnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) twice daily for eight weeks. The primary outcomes were changes in depression, anxiety, and stress levels measured by the Depression, Anxiety, and Stress Scale 42 (DASS-42). The secondary outcome was serum serotonin levels.

Results: The intervention group showed a significant decrease in total DASS-42 scores (p = 0.001) after eight weeks, indicating an overall reduction in psychological distress. However, changes in the scores of the subscales of the DASS-42 were not statistically significant (p > 0.05). Finally, serum serotonin levels increased in the intervention group, but the change was not statistically significant (p = 0.382).

Conclusion: An eight-week probiotic supplementation significantly reduced overall psychological symptoms, as shown by total DASS-42 scores, but did not lead to significant changes in depression, anxiety, or stress subscale scores. Serum serotonin levels also increased in the intervention group, though not significantly.

Keywords: probiotics, gut–brain axis, psychology, serotonin, chemotherapy

3. Introduction

Psychological disorders are becoming increasingly prevalent among patients diagnosed with cancer, with conditions such as delirium, depression, adjustment disorders, anxiety, sexual dysfunction, and sleep disorders affecting 30%–40% of this population (Mastan et al., 2024; Ostovar et al., 2022). The incidence of psychiatric disorders is even higher among those at advanced cancer stages. Anxiety was more common (varying from 7% to 88%) than depression (ranging from 3% to 65.5%) among cancer patients with diverse forms residing in different Southeast Asian nations (Ostovar et al., 2022). Additionally, a study in one Indonesian hospital reported that 23% of patients undergoing chemotherapy experienced depression, 40% suffered from anxiety, and 21% had stress (Mastan et al., 2024). Despite the significant impact of these conditions on patients' quality of life, psychiatric disorders in cancer patients are often underdiagnosed and inadequately treated, leading to further deterioration in their overall wellbeing (Mastan et al., 2024; Ostovar et al., 2022; S et al., 2018).

Chemotherapy has been shown to disrupt the balance of gut microbiota, a condition known as gut dysbiosis (Deleemans et al., 2019). This disruption can lead to a reduction in the diversity and number of commensal bacteria, which, in turn, can negatively affect mood and cognitive function (Deleemans et al., 2019; Maddern et al., 2023). Research has demonstrated that changes in microbiota composition can influence the development of the psychological symptoms of conditions including depression and anxiety (Deleemans et al., 2019; Maddern et al., 2023). Animal studies have further demonstrated that gut microbiota play a critical role in regulating the pathway associated with depression (Deleemans et al., 2019), suggesting that gut microbiota may be a promising therapeutic target for psychological disorders among cancer patients.

Since psychological disorders are linked to low serotonin levels—a condition that can be exacerbated by gut dysbiosis—targeting the gut microbiota through oral probiotics presents a promising therapeutic approach (Merkouris et al., 2024; Zhang et al., 2023). Probiotics have traditionally been used to mitigate the gastrointestinal side-effects of chemotherapy, such as nausea and vomiting (Vivarelli et al., 2019). Recent studies have suggested that probiotics may additionally improve psychological wellbeing by influencing neurotransmitter pathways, including serotonin pathways, which are closely associated with mood regulation (Merkouris et al., 2024; Zhang et al., 2023). Specifically, probiotics containing *Lactobacillus rhamnosus* and

Lactobacillus helveticus have been shown to reduce symptoms of depression, enhance cognitive function, and balance key neurochemicals, including serotonin, epinephrine, and brain-derived neurotrophic factor (BDNF), in animal studies (Deleemans et al., 2019; Ye et al., 2022).

Despite these promising findings, existing researches have predominantly focused on the effects of probiotics on the physical side-effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) on psychological disorders in a human sample has never been studied. We aim to fill this gap by assessing the impact of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using serum serotonin levels as a potential biomarker for these effects.

4. Methods

This study was a randomised, double-blinded, placebo-controlled pilot trial conducted in the outpatient clinic of [redacted] in 2023. The trial was designed to assess the effect of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using serum serotonin levels as a biomarker.

5.1 Participants

Participants included cancer patients who were undergoing chemotherapy in [redacted] The only inclusion criterion was that patients had to be aged between 18 and 76 years. Exclusion criteria were patients who smoked or had used antibiotics during the week prior to our intervention. Notably, the optimal abstinence period for antibiotics before participating in a study assessing probiotics and gut microbiota can vary depending on the type of antibiotic used and the patient's metabolism. Therefore, the abstinence period is usually calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days (Armstrong, 2020). Hence, a one-week abstinence period preceding this study seemed reasonable to minimise the potential impact of residual antibiotics on gut microbiota and the study outcomes.

5.2 Sample Size

The sample size was determined using the minimum sample for a pilot clinical trial (Julious, 2005). A total of 61 patients were enrolled and randomised into the intervention (n = 30) and control (n = 31) groups, with an allocation ratio of 1:1.

5.3 Randomisation and Blinding

Patients were randomly allocated to either the intervention or control group using block randomisation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of [redacted]. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.

5.4 Intervention

The treatment was given to the patient upon their arrival. The intervention group received probiotics (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52 at a dose of 2×10^9 CFU) twice a day for eight weeks.

5.5 Outcomes

The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) at baseline and after the eight-week intervention. The secondary outcome was a change in serum serotonin levels after the eight weeks, measured using an enzyme-linked immunosorbent assay (ELISA).

5.6 Research Instruments

To assess the primary outcomes, the following instrument was used:

3) DASS-42

The DASS-42 is a 42-item questionnaire used to measure the severity of depression, anxiety, and stress in participants. The questionnaire consists of 42 assessment statements (Table 1) to assess depression (14 statements), anxiety (14 statements), and stress (14 statements). Participants were asked to score each statement as 0—*never*, 1—*sometimes*, 2—*often*, or 3—*very often*. Subscale scores were then summed to determine the depression, anxiety, and stress scale scores (Table 2).

Meanwhile, to assess the secondary outcome, the following instrument was used:

1) Serotonin measurement

Serum serotonin levels were measured using an ELISA, following the manufacturer's protocol. Blood samples were collected at baseline and after the intervention, and results were read using a microplate reader (EL x 800).

5.7 Statistical Methods

Continuous variables (age, DASS-42 score, and serum serotonin level) were summarised using mean ± standard deviation, while categorical (nominal and ordinal) variables (gender, marital status, highest educational, jobs, history of psychiatric illness, history of psychiatric treatment, and duration of cancer diagnosis) were presented as frequencies and percentages.

For nominal variables (gender, marital status, and jobs), between group comparisons were conducted using chi-square test. Meanwhile, Mann-Whitney test was used for ordinal variables (highest education and duration since cancer diagnosis).

For continuous variables, Shapiro–Wilk test was used to assess the normality of data distribution. Between-group comparisons were conducted using independent t-test if the data was normally distributed and Mann–Whitney U test if the data was not normally distributed. Within-group comparisons were conducted using paired t-test if the data was normally distributed and using Wilcoxon signed rank test if the data was not normally distributed. A p-value < 0.05 was considered statistically significant

5.8 Ethics

The study was registered at the Indonesian Clinical Research Registry (INA-CRR) with registration number 042024030706474KRGNHZ, and it was approved by the Health Research Ethics Committee of [redacted] (No. 1496/EC/KEPK-RSDK/2023). All participants provided written informed consent prior to participation. The trial was conducted according to the principles of the Declaration of Helsinki.

6. Results

3.1 Sample Characteristics

This study included 61 cancer patients who met the inclusion and exclusion criteria. The study sample was divided into two groups via randomisation, namely an intervention group and a control group. A total of two research subjects from the intervention group died during the trial, four subjects were hospitalised during the trial, three subjects did not receive their treatment, and eight subjects were lost to follow-up, so the intervention group comprised 13 subjects. Meanwhile, two subjects from the control group died during the trial, one subject dropped out, three subjects were hospitalised during the trial, four subjects did not receive their treatment, and 15 subjects were lost to follow-up, so the control group also comprised 13 subjects (Figure 1).

Table 3 shows the characteristics of the study sample for each group. In this study sample, the mean ages in the intervention and control groups were 54.46 and 49.08 years, respectively. The number of male and female patients was approximately equal in the intervention and control groups. Most of the study sample was married (84.6%), had high school as their highest level of education (30.7%), and worked (65%). All samples had no history of psychiatric treatment or previous psychiatric diagnoses. There were also no significant differences in their age, gender, marital status, educational level, employment status, and duration of cancer, either.

3.2 The Effect of Probiotics on Depression, Stress, and Anxiety

The results showed an insignificant decrease in depression ($p = 0.317$), anxiety ($p = 0.914$), and stress ($p = 0.581$) scores, while there was a significant decrease in total DASS-42 scores ($p = 0.001$), in the intervention group after receiving probiotics for eight weeks (Table 4). When comparing the control group with the intervention group, there were insignificant differences in depression, anxiety, and stress scores, but total DASS-42 scores significantly differed between the two groups ($p = 0.048$; Table 4).

3.3 The Effect of Probiotics on Serotonin

We found an increase in serotonin levels in the intervention group, but it was not statistically significant ($p = 0.382$; Table 5).

7. Discussion

Cancer patients undergoing chemotherapy frequently experience significant psychological distress, including depression, anxiety, and stress, which adversely affects their quality of life (Ostovar et al., 2022). Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence the psychological symptoms of depression, anxiety, and stress via the gut–brain axis (Sabit et al., 2023). A systematic review also reported the beneficial effects of probiotic supplementation measured using the Hamilton Depression Rating Scale in patients with psychiatric disorders (Amirani et al., 2020). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris et al., 2024; Potter et al., 2023; Ye et al., 2022; Zhang et al., 2023).

In our study, the administration of probiotics over eight weeks caused a significant decrease in total DASS-42 scores in the intervention group ($p = 0.001$), indicating an overall reduction in psychological distress. However, the decreases in the scores for each subscale (depression, anxiety, and stress) were not statistically significant ($p > 0.005$). These findings suggest that despite the beneficial effect of probiotics on overall psychological symptoms, as measured using total DASS-42 scores, the impact of probiotics on specific symptoms (depression, anxiety, and stress) may be more limited or may require a longer intervention to become significant.

Chemotherapy, radiotherapy, and immunotherapy have toxic effects that can lead to changes in gut microbiota, a reduction in gut commensal bacteria, and inflammation of the gastrointestinal tract (Deleemans et al., 2019; Fernandes et al., 2024). Gut dysbiosis, a disruption in the gut microbiota, can lead to increased gut permeability, allowing toxins to enter the bloodstream and activating pro-inflammatory cytokines (IL-6, II-1β, TNF-a, and C-reactive protein), while it may cause the hyperactivation of the hypothalamic–pituitary axis (HPA-axis) (Deleemans et al., 2019). These inflammatory conditions then lead to decreased levels of serotonin (5-HT) and BDNF (Deleemans et al., 2019). Both of these can, in turn, cause psychological and cognitive changes such as anxiety and depression, fatigue, memory impairment, and decision-making impairment (Lu et al., 2022; Maddern et al., 2023; Merkouris et al., 2024; Sabit et al., 2023).

The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study,

while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.0382$). The control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, there are certain external factors that may have influenced these results. One such factor is the potential consumption of ondansetron. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels (Gupta et al., 2014), potentially explaining the more pronounced changes observed in the control group.

In addition, a diet that includes amino acids such as tryptophan, a precursor of serotonin, can contribute to variations in serotonin levels (Jenkins et al., 2016; Mohajeri et al., 2015) since tryptophan-rich foods can increase serotonin synthesis. However, dietary habits were not controlled for in this study, even though differences in diets among participants might have affected the results. Future studies should thus control for the medications and diets of their participants more rigorously.

An increase in serotonin is one of several mechanisms involved in the improvement of depression, anxiety, and stress symptoms from probiotic use. In addition to serotonin production, probiotics may reduce symptoms of depression and anxiety by decreasing stress-induced HPA responses, decreasing cortisol levels, increasing neurotransmitter synthesis (GABA, dopamine, noradrenaline, melatonin, histamine, and acetylcholine), stimulating the production of gut neuropeptides (glucagons like peptide-1 and tyrosine), improving the gut barrier, increasing BDNF production, decreasing the release of pro-inflammatory cytokines, and increasing anti-inflammatory cytokines (Lu et al., 2022; Sabit et al., 2023; Zhang et al., 2023).

Overall, the gut–brain axis and serotonin production are influenced by numerous factors beyond just probiotic supplementation, including stress levels, diet, and physical activity (Lou et al., 2023; Madison & Kiecolt-Glaser, 2019; Mohajeri et al., 2015). The influence of these variables might have differed between the control and intervention groups, contributing to the outcomes seen in our study.

4.1 Limitations

This study had several limitations. Specifically, we used serum serotonin as a biomarker. While the direct impact of central nervous system (CNS) serotonin on the pathophysiology of depression, anxiety, and stress is well-recognised, the direct measurement of CNS serotonin is invasive and not feasible in a clinical trial setting. Therefore, in this study, we used serum serotonin levels as a biomarker to measure the potential impact of probiotic supplementation on serotonin levels. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may not accurately reflect CNS serotonin concentration. Although this was a limitation of our methodology, it is considered a feasible and ethical non-invasive sampling method for human subjects. Moreover, serum serotonin remains a valuable biomarker in studies exploring the gut–brain axis and the systemic effects of probiotic supplementation (Merkouris et al., 2024). This is because previous studies have reported that gut microbiota can modulate systemic serotonin levels, which, in turn, may influence CNS function through the gut–brain axis (Jenkins et al., 2016; Potter et al., 2023; Yano et al., 2015).

Another limitation of this study was the brevity of the intervention. Eight weeks may not have been sufficient to observe significant changes in the psychological conditions of depression, anxiety, and stress. Studies have suggested that gut microbiota exist in a complex and dynamic ecosystem that may require longer probiotic interventions to undergo significant alterations (Ng et al., 2023). Another study also reported the beneficial effects of probiotic supplementation on symptoms of depression after 12 weeks among patients under methadone maintenance treatment programmes (Molavi et al., 2022).

Additionally, we did not control for physical activity, dietary habits, symptomatic medications, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients.

Based on these limitations, future research should explore the effects of longer-term probiotic supplementation on the psychological symptoms of cancer patients undergoing chemotherapy and control for external factors more rigorously, perhaps through more detailed dietary assessments, physical activity logs, and the closer monitoring of medication use.

8. Conclusions

We found that an eight-week probiotic supplementation regimen significantly decreases overall psychological symptoms, as measured by total DASS-42 scores. However, we found no statistically significant changes in the depression, anxiety, and stress subscale scores of participants. In addition, even though the serum serotonin levels in the intervention group increased, this alteration was not statistically significant. Hence, the results of this pilot study show the promise of probiotic supplementation for psychological symptoms among cancer patients and should be extended with longer intervention periods, larger sample sizes, and stricter inclusion and exclusion criteria, in addition to controlling for other influencing factors.

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Table 1. Statements for the depression, anxiety, and stress subscales

Subscale	Statement number
Depress	3, 5, 10, 13, 16, 17, 21, 24, 26, 31, 34, 37, 38, 42
Anxiety	2, 4, 7, 9, 15, 19, 20, 23, 25, 28, 30, 36, 40, 41
Stress	1, 6, 8, 11, 12, 14, 18, 22, 27, 29, 32, 33, 35, 39

Table 2. DASS-42 interpretation

	Depression	Anxiety	Stress
Normal	0–9	0–7	0–14
Mild	10–13	8–9	15–18
Moderate	14–20	10–14	19–25
Severe	21–27	15–19	26–33
Very Severe	≥ 28	≥ 20	≥ 34

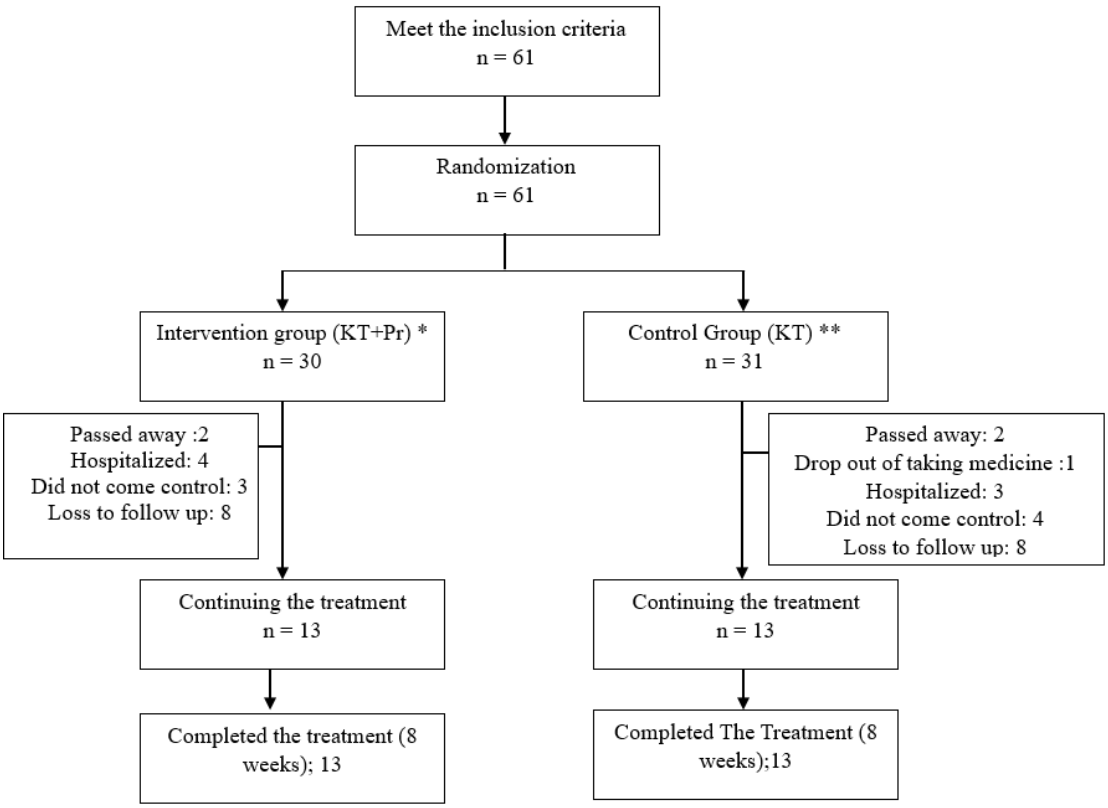

Figure 1. CONSORT diagram. This study included 61 cancer patients, randomised into an intervention group and a control group.

Description:

*KT + Pr = Chemotherapy + probiotics

**KT = Chemotherapy + placebo

Table 3. Characteristics of the research sample

Variables	**Intervention (*n* = 13)**	**Control (*n* = 13)**	***p*-value***
Age			
Mean ± SD	54 ± 7.88	49 ± 17.06	0.079[§]
Median (min–max)	54 (43–68)	54 (23–76)	
	Frequency (%)		
Variables	**Intervention (*n* = 13)**	**Control (*n* = 13)**	***p*-value**

Gender			
Male	6 (46.2%)	6 (46.2%)	1.000¥
Female	7 (53.8%)	7 (53.8%)	
Marital status			
Not married	1 (7.7%)	2 (15.4%)	0.703¥
Married	11 (84.6%)	11 (84.6%)	
Divorced	1 (7.7%)	0 (0%)	
Highest education			
Elementary school	1 (7.7%)	1 (7.7%)	0.710‡
Junior high school	2 (15.4%)	4 (30.8%)	
Senior high school	5 (38.5%)	3 (23.1%)	
Bachelor	4 (30.8%)	4 (30.8%)	
Did not attend school	1 (7.7%)	1 (7.7%)	
Jobs			
Working	8 (61.5%)	9 (69.2%)	0.500¥
Not working	5 (38.5%)	4 (30.8%)	
History of psychiatric treatment (including benzodiazepine)			
Yes	0	0	.[e]
No	13 (50%)	13 (50%)	
Psychiatric diagnosis			
Yes	0	0	.[e]
No	13 (50%)	13 (50%)	
Duration of psychiatric treatment			
Yes	0	0	.[e]
No	13 (50%)	13 (50%)	
Duration since cancer diagnosis			
3–6 months	2 (15.4%)	4 (30.8%)	0.518‡
6 months–1 year	5 (38.5%)	4 (30.8%)	

1–5 years	5 (38.5%)	4 (30.8%)
> 5 years	1 (7.7%)	1 (7.7%)

‡Mann–Whitney; §independent t; ¥chi-square; ᵉnot measurable because $n = 0$, *$p < 0.05$ was considered statistically significant

Table 4. Comparison of depression scores for the intervention and control groups before and after the intervention

DASS-42	Group		*p*
	Intervention	**Control**	
Pre-intervention	19.00 ± 7.10	13.69 ± 7.06	0.068§
Post-intervention	17.38 ± 6.48	11.15 ± 6.83	0.048‡*
p	0.001¶*	0.002†*	0.207§
Depression			
Pre-intervention	5.89 ± 3.20	6.31 ± 3.77	0.658§
Post-intervention	5.46 ± 3.05	4.69 ± 2.78	0.508§
p	0.317†	0.010†*	0.058‡
Anxiety			
Pre-intervention	5.23 ± 3.86	5.15 ± 8.16	0.188‡
Post-intervention	5.46 ± 4.05	3.54 ± 4.82	0.055‡
p	0.914†	0.024†*	0.081‡
Stress			
Pre-intervention	6.15 ± 3.02	9.15 ± 5.54	0.099§
Post-intervention	6.46 ± 2.33	6.92 ± 4.27	0.735§
p	0.581†	0.007†*	0.003‡

* Statistically significant ($p < 0.05$); ‡ Mann–Whitney; § independent t; ¶ paired t; † Wilcoxon

Table 5. Comparison of serotonin levels between the intervention and control groups before and after the intervention

Serotonin	Group		*p*
	Intervention	**Control**	
Pre-intervention	98.85 ± 125.22	145.77 ± 199.78	0.798‡
Post-intervention	104.15 ± 195.69	161.38 ± 175.37	0.012‡*
p	0.382†	0.087†	
Difference	5.31 ± 77.48	15.62 ± 66.20	0.048‡*

*Statistically significant ($p < 0.05$); ‡ Mann–Whitney; † Wilcoxon

Third revision (01-10-2024)

From: **Mental Health & Prevention** <em@editorialmanager.com>
Date: Tue, 1 Oct 2024, 10:41
Subject: Decision on submission to Mental Health & Prevention
To: Alifiati Fitrikasari <fitrisutomo@yahoo.com>

Manuscript Number: **MHP-D-24-00222R2**

Probiotic Supplementation Effects on Depression, Anxiety, and Stress in Cancer Patients on Chemotherapy via Serotonin Biomarker Analysis: A Pilot Trial

Dear Mrs Fitrikasari,

Thank you for submitting your revised manuscript to Mental Health & Prevention. I have completed my evaluation of your manuscript, and two reviewers completed further reviewer. The reviewers recommend reconsideration of your manuscript following major revision.

I invite you to resubmit your manuscript after addressing the reviewer comments below. In addition, I invite you to consider the following:

- I note that the authors refer to your study as a pilot study. If this is the case, then the primary aim is to assess feasibility (and acceptability, if you included measures for this). This typically means that you are not powered to determine intervention effects/benefit or effectiveness (which has a different meaning). This has implications for the language you use throughout to discuss results - it is not possible to refer to the 'impact', 'effects', 'outcomes' or 'efficacy' of your intervention. You cannot talk about whether there are 'significant differences' in your intervention versus control group. I recommend a thorough reading of this resource: https://www.nccih.nih.gov/grants/pilot-studies-common-uses-and-misuses.
- If you are adequately powered to investigate intervention effects, please include a sample size calculation in the paper.

Please resubmit your revised manuscript by **Oct 15, 2024**.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

To submit your revised manuscript, please log in as an author at https://www.editorialmanager.com/mhp/, and navigate to the "Submissions Needing Revision" folder.

Research Elements (optional)
This journal encourages you to share research objects - including your raw data, methods, protocols, software, hardware and more – which support your original research article in a Research Elements journal. Research Elements are open access, multidisciplinary, peer-reviewed journals which make the objects associated with your research more discoverable, trustworthy and promote replicability and reproducibility. As open access journals, there may be an Article Publishing Charge if your paper is accepted for publication. Find out more about the Research Elements journals at https://www.elsevier.com/authors/tools-and-resources/research-elements-journals?dgcid=ec_em_research_elements_email.

Mental Health & Prevention values your contribution and I look forward to receiving your revised manuscript.

Kind regards,
Elizabeth Westrupp
Deputy Editor

Third revision submitted (14-10-2024)

From: Mental Health & Prevention <em@editorialmanager.com>
To: Alifiati Fitrikasari <fitrisutomo@yahoo.com>
Sent: Monday, October 14, 2024 at 10:37:35 AM GMT+7
Subject: Confirming submission to Mental Health & Prevention

This is an automated message.

Manuscript Number: **MHP-D-24-00222R3**

Probiotic Supplementation for Reducing Psychological Symptoms in Cancer Patients on Chemotherapy: a Pilot Trial

Dear Mrs Fitrikasari,

We have received the above referenced manuscript you submitted to Mental Health & Prevention.

To track the status of your manuscript, please log in as an author at https://www.editorialmanager.com/mhp/, and navigate to the "Revisions Being Processed" folder.

Thank you for submitting your revision to this journal.

Kind regards,
Mental Health & Prevention

Revisions and Amends

Points-by-Points Revision

Manuscript: Fitrikasari et al. “Probiotic Supplementation for Reducing Psychological Symptoms in Cancer Patients on Chemotherapy: a Pilot Trial”

Dear Editor in Chief and reviewers,

We really appreciate the constructive feedback you have provided in the second-round review of our manuscript. We attempted to address all suggestions and comments meticulously. As suggested, we have formatted the revision so that the changes can be tracked. The details of the revision are as follow:

DEPUTY EDITOR			
No	Review	Revision	Page
1	I note that the authors refer to your study as a pilot study. If this is the case, then the primary aim is to assess feasibility (and acceptability, if you included measures for this). This typically means that you are not powered to determine intervention effects/benefit or effectiveness (which has a different meaning). This has implications for the language you use throughout to discuss results - it is not possible to refer to the 'impact', 'effects', 'outcomes' or 'efficacy' of your intervention. You cannot talk about whether there are 'significant differences' in your intervention versus control group. I recommend a thorough reading of this resource: https://www.nccih.nih.gov/grants/pilot-studies-common-uses-and-misuses. If you are adequately powered to investigate intervention effects, please include a sample size calculation in the paper.	Thank you for your correction. We have read the link you suggested and revised several parts in the manuscript, especially the discussion section, to emphasize the feasibility of the study, not the effect of the intervention, since it is a pilot study. Introduction section Despite these promising findings, existing researches have predominantly focused on the effects of probiotics on the physical side-effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (Lactobacillus rhomnosus Rosell-11 and Lactobacillus helveticus Rosell-52) on psychological disorders in a human sample has never been studied. Therefore, this pilot study aims to assess the feasibility and acceptability of probiotic supplementation to reduce psychological symptoms in cancer patients undergoing chemotherapy. The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) and the secondary outcome was a change in serum serotonin levels. Discussion section While probiotics have been shown to influence the gut-brain axis and improve psychological symptoms in other contexts, their effects on cancer patients undergoing chemotherapy are not well-established. Thus, this study provides preliminary insights into the feasibility and potential efficacy of probiotics in reducing psychological symptoms in this population.	Page 3, line 61-70 Page 7, line 203-207

		The results indicate that the administration of probiotics over eight weeks caused a significant decrease in total DASS-42 scores ($p = 0.001$), suggesting an overall reduction in psychological distress. However, as a pilot study, these findings are exploratory and should be interpreted with caution. The observed number needed to treat (NNT) of 9 for total DASS-42 score reduction demonstrates that the intervention may have clinical relevance, but this needs confirmation in larger trials. In terms of the specific DASS subscales (depression, anxiety, and stress), the decreases in the scores for each subscale were not statistically significant ($p > 0.005$). The most notable effect was seen in the stress subscale, with an NNT of 5, showing that probiotics may have a more positive effect on stress symptoms in cancer patients. However, the larger NNTs for the depression (8) and anxiety (17) subscales exhibit the need for further investigation with larger sample sizes to determine the true effect of probiotic supplementation on these specific psychological symptoms. The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.38$) and the NNT of 7 should be viewed as an exploratory finding. Conclusion This pilot study provides preliminary evidence that eight weeks of probiotic supplementation may have a potential role in reducing overall psychological symptoms in cancer patients undergoing chemotherapy, as shown by changes in total DASS-42 scores. However, the results should be interpreted carefully due to the small sample size, high dropout rate, and limitations associated with other factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Future larger trials with more rigorous controls and longer intervention periods are needed to confirm these preliminary findings and to further explore the therapeutic potential of probiotics on psychological symptoms in cancer patients undergoing chemotherapy.	Page 8, line 212-223 Page 8, line 235-239 Page 11, line 310-318

REVIEWER 1			
No	Review	Revision	Page
Title			
1	The study title can be more concise and clearer, for example, "Probiotic Supplementation for Reducing Psychological Symptoms in Chemotherapy Patients: A Pilot Trial."	Thank you for the suggestion. We have revised the title to be more concise and clearer. Probiotic Supplementation for Reducing Psychological Symptoms in Cancer Patients on Chemotherapy: a Pilot Trial	Title page
Introduction			
1	Suggest to state the primary and secondary objectives more clearly upfront.	Thank you for your suggestion. We have clearly stated the primary and secondary outcomes of our study in the introduction section. The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) and the secondary outcome was a change in serum serotonin levels.	Page 3, line 68-70
Methods			
1	I am not sure the value of having Tables 1 and 2 in the main manuscript. They can easily be moved to the supplementary or summarised in a few sentences. It should also be "Depression" subscale and not "Depress".	Thank you very much for the recommendation. We have summarized the explanation of DASS-42 questionnaire in a few sentences. The DASS-42 is a 42-item questionnaire used to measure the severity of depression, anxiety, and stress in participants. The questionnaire consists of 42 assessment statements (supp 1) to assess depression (14 statements), anxiety (14 statements), and stress (14 statements). Participants were asked to score each statement as 0—never, 1—sometimes, 2—often, or 3—very often. Subscale scores were then summed to determine the depression, anxiety, and stress scale scores (supp 1).	Page 4, line 114-119
2	I am not sure why the authors even chose to measure peripheral serotonin at all. It is well-known that serotonin metabolism is deranged in cancer patients given that treatments can induce marked increases in serotonin release. Chemotherapeutic agent can cause a substantial release of serotonin from enterochromaffin cells in the gut. This release is not directly related to the gut-brain axis but is a consequence of	Thank you for your suggestion. We have revised the limitation section to consider serotonin as a biomarker carefully for further studies. A major concern raised by reviewers involves our choice of serum serotonin as a biomarker. While the direct impact of central nervous system (CNS) serotonin on the pathophysiology of depression, anxiety, and stress is well-recognised, the direct measurement of CNS serotonin is invasive and not feasible in a clinical trial setting. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may not accurately reflect CNS serotonin concentration. Although this was a limitation of our methodology, it is considered a feasible and ethical non-invasive sampling method for human subjects. Thus, many studies have already used this biomarker to investigate the systemic effects of probiotic supplementation (Jenkins et al., 2016; Merkouris et al., 2024; Potter et al., 2023; Yano et al., 2015). However, chemotherapy can induce serotonin release from enterochromaffin cells in the gut (Cubeddu et al., 1995). This drug-induced serotonin release could have confounded the effects of probiotics.	Page 10, line 279-291

	gastrointestinal toxicity and mucosal damage induced by these treatments. Such drug-induced serotonin fluctuations can mask or confound the effects of probiotics on serotonin modulation, making the results difficult to interpret. As such, it would be prudent to consider other markers such as IL-6 or TNF-alpha or ensure tighter control over confounding variables like chemotherapy type, anti-emetic use, etc.	To mitigate this in future studies, alternative biomarkers should be utilized and other potential confounders such as chemotherapy type, anti-emetic use, and dietary factors should be controlled.	
3	For the statistical analysis, please mention the software (and version number and packages if applicable) used.	Thank you for asking. This research used IBM SPSS Statistics 20 version for statistical analysis.	
4	Authors should consider performing an intention-to-treat (ITT) analysis, which includes all randomized patients in the groups to which they were originally assigned. This might help handle the dropouts.	Thank you for your recommendation. We have conducted the intention-to-treat analysis and reported it along the manuscript. Method section An intention-to-treat (ITT) analysis was also performed to assess the effect of the intervention on several outcomes, including the total DASS score, it's subscales (depressions, anxiety, and stress), and serotonin level. All participants were analyzed based on control and intervention groups regardless of whether they completed the study or not. The intended outcomes were the decrease in total DASS score and it's subscales, and increase in serotonin levels. The calculated metrics were control event rate (CER), experimental event rate (EER), absolute risk reduction (ARR), relative risk reduction (RRR), and number needed to treat (NNT). Result section 3.3 Intention-to-Treat (ITT) Analysis As presented in supp 3, the CER, EER, ARR, RRR, and NNT provide early indicators of the intervention's potential impact. The results demonstrate a beneficial effect of probiotics in reducing overall psychological symptoms, as evidenced by increasing of total DASS-42 scores. However, the effect sized for the depression, anxiety, and stress subscales, as well as serotonin levels, were smaller and not statistically significant. The NNT value, despite exploratory, suggest that approximately nine patients would need to be treated with probiotics to achieve a reduction in total psychological symptoms in one patient. Discussion section	Page 5, line 140-146 Page 7, line 183-190

<table>
<tr><td></td><td></td><td>The intention-to-treat (ITT) analysis was crucial in this pilot study to account for all randomized participants, including those who did not complete the study. The ITT approach provides a more realistic estimation of the treatment effect, especially in the study which has high dropout rate (Ahn & Kang, 2023).
The results indicate that the administration of probiotics over eight weeks caused a significant decrease in total DASS-42 scores ($p = 0.001$), suggesting an overall reduction in psychological distress. However, as a pilot study, these findings are exploratory and should be interpreted with caution. The observed number needed to treat (NNT) of 9 for total DASS-42 score reduction demonstrates that the intervention may have clinical relevance, but this needs confirmation in larger trials.
In terms of the specific DASS subscales (depression, anxiety, and stress), the decreases in the scores for each subscale were not statistically significant ($p > 0.005$). The most notable effect was seen in the stress subscale, with an NNT of 5, showing that probiotics may have a more positive effect on stress symptoms in cancer patients. However, the larger NNTs for the depression (8) and anxiety (17) subscales exhibit the need for further investigation with larger sample sizes to determine the true effect of probiotic supplementation on these specific psychological symptoms.
The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.38$) and the NNT of 7 should be viewed as an exploratory finding.</td><td>Page 7-8, line 208-223

Page 8, line 235-239</td></tr>
<tr><td>5</td><td>Although the authors mentioned that block randomization with a block size of four was used, it is unclear whether any stratification was done to ensure balance on key variables (e.g., patient's age, cancer stage, type of chemotherapy). This makes it difficult to assess whether the randomization process was sufficient to prevent biases in treatment allocation</td><td>Thank you for your comment. We did not conduct stratification because the pilot nature of the study and the relatively small sample size. Thus, we prioritized a straightforward randomization procedure. We understand that stratification might minimize potential biases even further, however we belief that the randomization process is adequate to ensure balanced treatment allocation across the intervention and control groups.
In addition, we already mentioned in the result section that baseline characteristics, including age, cancer diagnosis, duration since cancer diagnosis, pre-intervention DASS score and serotonin level were comparable between the intervention and control groups, indicating that the randomization process effectively reduced potential biases in treatment allocation.

The baseline characteristics of the participants in the intervention (n=30) and control (n=31) groups are shown in supplementary file. There were no significant differences between the two groups for any of the baseline characteristics (age, cancer diagnosis, duration since cancer diagnosis, baseline DASS scores, and baseline serotonin level).</td><td>Supp file table 3 & 4,
Page 6, line 158-160</td></tr>
<tr><td colspan="4">Results</td></tr>
<tr><td>1</td><td>There should be a table comparing baseline characteristics (e.g., patient's age, cancer type, stage of cancer, chemotherapy type, baseline DASS-42 scores, serotonin levels) between the two groups. This helps assess whether the</td><td>Thank you for your suggestion. We have provided a table comparing baseline characteristics between control and intervention group in supplementary file. Based on the statistical analysis, there were no significant differences between the two groups for any of the baseline characteristics.

Table 3. Baseline Comparison of Intervention and Control Group
<table>
<tr><th>Characteristics</th><th>Intervention (n=30)</th><th>Control (n = 31)</th><th>p-value*</th></tr>
<tr><td>Age</td><td>49 ± 11.26</td><td>48 ± 13.62</td><td>0.63§</td></tr>
</table></td><td>Supp file</td></tr>
</table>

	randomization process successfully created balanced groups.	Duration since cancer diagnosis				
		<3 months	5 (16.7%)	1 (3.2%)	0.45¥	
		3-6 months	6 (20%)	10 (32.3%)		
		6 months - 1 year	9 (30%)	9 (29%)		
		1-5 years	9 (30%)	10 (32.3%)		
		>5 years	1 (3.3%)	1 (3.2%)		
		Cancer diagnosis			0.62¥	
		breast cancer	0	1 (3.2%)		
		lung cancer	2 (6.7%)	2 (6.5%)		
		colorectal cancer	13 (43.3%)	9 (29%)		
		prostate cancer	1(3.3%)	1 (3.2%)		
		gastrointestinal	2 (6.7%)	4 (12.9%)		
		gynaecological cancer	2 (6.7%)	4 (12.9%)		
		haematological cancer	5(16.7%)	8 (25.8%)		
		head and neck cancer	0	1 (3.2%)		
		skin cancer	2 (6.7%)	0		
		urinary cancer	2(6.7%)	1 (3.2%)		
		other	1(3.3%)	0		
		Baseline DASS score	19 ± 11.79	20 ± 14.48	0.68§	
		Baseline serotonin level	(n=28)* 113 ± 154.93	(n=27)* 117 ± 149.51	0.94§	
		§independent t; ¥chi-square; *the baseline serotonin level was not measured in patients who passed away and stopped taking medicine				
2	Given the high dropout rate, the reasons for dropout (e.g., death or hospitalization) should be more clearly detailed and addressed in terms of how they might have impacted the results. In the supplementary, it would also be useful to have a table summarizing the characteristics of those who dropped out versus those who completed the study would be useful. This would help identify whether there were any systematic differences between completers and non-completers that could bias the results towards or away from the null.	Thank you for your suggestion. We have provided a table summarizing the characteristics between those who dropped out versus those who completed the study in supplementary file. We also mentioned the reasons for dropout. Based on the statistical analysis, there were no significant differences between the two groups for any of the characteristics.				Supp file
		Table 4. Comparison between Dropouts vs Completers				
		Characteristics	**Dropped Out (n=35)**	**Completed (n = 26)**	***p*-value***	
		Age	46 ± 11.4	52 ± 13.3	0.09§	
		Duration since cancer diagnosis			0.095¥	
		<3 months	6 (17.1%)	0		
		3-6 months	10 (28.6%)	6 (23.1%)		
		6 months - 1 year	9 (25.7%)	9 (34.6 %)		
		1-5 years	10 (28.6%)	9 (34.6%)		
		>5 years	0	2 (7.7%)		
		Cancer diagnosis			0.75¥	
		breast cancer	1 (2.9%)	0		

		lung cancer	3 (8.6%)	1 (3.8%)		
		colorectal cancer	12 (34.3%)	10 (38.5%)		
		prostate cancer	2 (5.7%)	0		
		gastrointestinal	4 (11.4%)	2 (7.7%)		
		gynaecological cancer	2 (5.7%)	4 (15.4%)		
		haematological cancer	7 (20%)	6 (23.1%)		
		head and neck cancer	1 (2.9%)	0		
		skin cancer	1 (2.9%)	1 (3.8%)		
		urinary cancer	2 (5.7%)	1 (3.8%)		
		other	0	1 (3.8%)		
		Baseline DASS score	20 ± 14.05	19 ± 12.03	0.71§	
		Baseline serotonin level	(n=29)* 108 ± 139.56	(n=26) 122 ± 165.1	0.74§	
		Reason for dropout				
		hospitalized	7 (20%			
		did not come control	7 (20%)			
		unresponsive	16 (45.7%)			
		passed away	4 (11.4%)			
		stop taking medicine	1 (2.9%)			

§independent t; ¥chi-square; *the baseline serotonin level was not measured in patients who passed away and stopped taking medicine

The revision of result section

The primary reasons for dropout included unresponsiveness to contact attempts (45.7%), hospitalisation (20%), failure to attend control visits (20%), and death (11.4%) (supp 2). Those who were unresponsive were participants whom the research team was unable to contact, despite repeated follow-up attempts. As a result, the intervention group comprised 13 subjects, so did the control group (figure 1).

Page 6, line 161-165

The revision in discussion section

The high dropout rate was primarily driven by factors such as unresponsiveness, hospitalization, and death. Most of the dropout patients comprised individuals who were unresponsive to follow-up attempts (45.7%). Despite repeated attempts to contact them by telephone or messengers, participants could not be reached. Given that the comparison data of baseline characteristics between dropout and completer groups showed no significant differences, the risk of selection bias is minimized. However, the high dropout rate still poses a limitation to the statistical power, because the small sample size and homogeneity of the study population may limit the generalizability of the findings. Further larger-scale study should aim to implement strategies to improve participant retention, such as a more flexible follow-up options or using alternative contact methods, and include more diverse population to increase the generalizability of the findings.

Page 9-10, line 268-278

3	With at least four DASS-42 outcomes (total score, depression, anxiety, stress) and	Thank you for your suggestions. Since we did not control for potential inflation of Type I error, we put it under study limitation.	

	the serotonin outcome, there should have been a correction applied to control for the potential inflation of Type I error.	Another important limitation is that we did not apply any statistical correction to control for the potential inflation of Type I error because we conducted multiple comparisons. In future studies, statistical corrections should be applied when analyzing multiple outcomes.	Page 10, line 292-294
4	When reporting the results, the authors report some values with excessive precision, such as two decimal places for mean ages and p-values (e.g., "p = 0.382"). This level of precision is not meaningful and can be misleading, especially given the small sample size. Descriptive statistics should be rounded appropriately to avoid giving a false impression of accuracy. 95% CIs should also be reported alongside p-values, wherever applicable.	Thank you for your recommendation. We have decreased the number of decimals for the data. We also reported the 95% CI along p-value if it is appropriate. **Table 2.** Comparison of depression scores for the intervention and control groups before and after the intervention	Page 16, line 437

DASS-42	Group		*p*
	Intervention	Control	
Pre-intervention	19.00 ± 7.10	13.69 ± 7.06	0.068§ (95% CI -0.42, 11.04)
Post-intervention	17.38 ± 6.48	11.15 ± 6.83	0.048‡*
p	0.001¶* (95% CI 0.78, 2.45)	0.002†*	0.207§
Depression			
Pre-intervention	5.69 ± 3.20	6.31 ± 3.77	0.658§ (95% CI -3.4, 2.2)
Post-intervention	5.46 ± 3.05	4.69 ± 2.78	0.508§ (95% CI -1.6, 3.1)
p	0.317†	0.010†*	0.058‡
Anxiety			
Pre-intervention	5.23 ± 3.86	5.15 ± 8.16	0.188‡
Post-intervention	5.46 ± 4.05	3.54 ± 4.82	0.055‡
p	0.914†	0.024†*	0.081‡
Stress			
Pre-intervention	6.15 ± 3.02	9.15 ± 5.54	0.099§ (95% CI -6.6, 0.6)
Post-intervention	6.46 ± 2.33	6.92 ± 4.27	0.735§ (95% CI -3.2, 2.3)
p	0.581†	0.007†*	0.003‡

* Statistically significant ($p < 0.05$); ‡ Mann–Whitney; § independent t; ¶ paired t; † Wilcoxon

Discussion			

1	Although the authors did list some limitations, such as the brevity of the intervention and lack of control over lifestyle factors, they do not fully address the impact of the high dropout rate, potential selection bias, and the limited generalizability of the findings due to the small sample size and homogeneity of the population.	Thank you for your suggestion. We have added the high dropout rate, potential selection bias, and limited generalizability in the limitation section. We also have given several recommendations for further studies. The high dropout rate was primarily driven by factors such as unresponsiveness, hospitalization, and death. Most of the dropout patients comprised individuals who were unresponsive to follow-up attempts (45.7%). Despite repeated attempts to contact them by telephone or messengers, participants could not be reached. Given that the comparison data of baseline characteristics between dropout and completer groups showed no significant differences, the risk of selection bias is minimized. However, the high dropout rate still poses a limitation to the statistical power, because the small sample size and homogeneity of the study population may limit the generalizability of the findings. Further larger-scale study should aim to implement strategies to improve participant retention, such as a more flexible follow-up options or using alternative contact methods, and include more diverse population to increase the generalizability of the findings.	Page 9-10, line 268-278
2	The conclusion that probiotic supplementation "significantly reduced overall psychological symptoms" seems overstated given the preliminary nature of the study and the numerous limitations. Please temper this accordingly.	Thank you for your recommendation. We have revised the conclusion section to make it more suitable with the nature of pilot study. This pilot study provides preliminary evidence that eight weeks of probiotic supplementation may have a potential role in reducing overall psychological symptoms in cancer patients undergoing chemotherapy, as shown by changes in total DASS-42 scores. However, the results should be interpreted carefully due to the small sample size, high dropout rate, and limitations associated with other factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Future larger trials with more rigorous controls and longer intervention periods are needed to confirm these preliminary findings and to further explore the therapeutic potential of probiotics on psychological symptoms in cancer patients undergoing chemotherapy.	Page 11, line 310-318

Revised version

ABSTRACT

Background: Psychological disorders, including depression, anxiety, and stress, are prevalent among cancer patients undergoing chemotherapy. Probiotics have been investigated as a potential supplement to modulate the gut–brain axis and improve psychological symptoms, possibly through mechanisms such as serotonin regulation. However, studies on the effects of probiotics on psychological symptoms in chemotherapy patients are limited.

Methods: This randomised, double-blinded, placebo-controlled pilot trial was conducted at the outpatient clinic [redacted], in 2023. Sixty-one cancer patients undergoing chemotherapy were enrolled and randomised into an intervention (n = 30) and control (n = 31) group. The intervention group received probiotics (*Lactobacillus rhamnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) twice daily for eight weeks. The primary outcomes were changes in depression, anxiety, and stress levels measured by the Depression, Anxiety, and Stress Scale 42 (DASS-42). The secondary outcome was serum serotonin levels.

Results: The intervention group showed a significant decrease in total DASS-42 scores (p = 0.001), indicating an overall reduction in psychological distress. However, changes in the scores of the subscales (depression, anxiety, and stress) were not statistically significant ($p > 0.05$). Serum serotonin levels increased in the intervention group, but this was not statistically significant (p = 0.38). The findings should be interpreted cautiously due to small sample size and potential confounding factors.

Conclusion: This pilot study suggests that eight weeks of probiotic supplementation may reduce overall psychological symptoms in cancer patients undergoing chemotherapy. Larger trials with rigorous controls and longer interventions are needed to confirm these preliminary findings.

Keywords: probiotics, gut–brain axis, psychology, serotonin, chemotherapy

5. Introduction

Psychological disorders are becoming increasingly prevalent among patients diagnosed with cancer, with conditions such as delirium, depression, adjustment disorders, anxiety, sexual dysfunction, and sleep disorders affecting 30%–40% of this population (Mastan et al., 2024; Ostovar et al., 2022). The incidence of psychiatric disorders is even higher among those at advanced cancer stages. Anxiety was more common (varying from 7% to 88%) than depression (ranging from 3% to 65.5%) among cancer patients with diverse forms residing in different Southeast Asian nations (Ostovar et al., 2022). Additionally, a study in one Indonesian hospital reported that 23% of patients undergoing chemotherapy experienced depression, 40% suffered from anxiety, and 21% had stress (Mastan et al., 2024). Despite the significant impact of these conditions on patients' quality of life, psychiatric disorders in cancer patients are often underdiagnosed and inadequately treated, leading to further deterioration in their overall wellbeing (Mastan et al., 2024; Ostovar et al., 2022; S et al., 2018).

Chemotherapy has been shown to disrupt the balance of gut microbiota, a condition known as gut dysbiosis (Deleemans et al., 2019). This disruption can lead to a reduction in the diversity and number of commensal bacteria, which, in turn, can negatively affect mood and cognitive function (Deleemans et al., 2019; Maddern et al., 2023). Research has demonstrated that changes in microbiota composition can influence the development of the psychological symptoms of conditions including depression and anxiety (Deleemans et al., 2019; Maddern et al., 2023). Animal studies have further demonstrated that gut microbiota play a critical role in regulating the pathway associated with depression (Deleemans et al., 2019), suggesting that gut microbiota may be a promising therapeutic target for psychological disorders among cancer patients.

Since psychological disorders are linked to low serotonin levels—a condition that can be exacerbated by gut dysbiosis—targeting the gut microbiota through oral probiotics presents a promising therapeutic approach (Merkouris et al., 2024; Zhang et al., 2023). Probiotics have traditionally been used to mitigate the gastrointestinal side-effects of chemotherapy, such as nausea and vomiting (Vivarelli et al., 2019). Recent studies have suggested that probiotics may additionally improve psychological wellbeing by influencing neurotransmitter pathways, including serotonin pathways, which are closely associated with mood regulation (Merkouris et al., 2024; Zhang et al., 2023). Specifically, probiotics containing *Lactobacillus rhamnosus* and

Lactobacillus helveticus have been shown to reduce symptoms of depression, enhance cognitive function, and balance key neurochemicals, including serotonin, epinephrine, and brain-derived neurotrophic factor (BDNF), in animal studies (Deleemans et al., 2019; Ye et al., 2022).

Despite these promising findings, existing researches have predominantly focused on the effects of probiotics on the physical side-effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) on psychological disorders in a human sample has never been studied. Therefore, this pilot study aims to assess the feasibility and acceptability of probiotic supplementation to reduce psychological symptoms in cancer patients undergoing chemotherapy. The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) and the secondary outcome was a change in serum serotonin levels.

6. Methods

This study was a randomised, double-blinded, placebo-controlled pilot trial conducted in the outpatient clinic of [redacted] in 2023. The trial was designed to assess the effect of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using serum serotonin levels as a biomarker.

8.1 Participants

Participants included cancer patients who were undergoing chemotherapy in [redacted] The only inclusion criterion was that patients had to be aged between 18 and 76 years. Exclusion criteria were patients who smoked or had used antibiotics during the week prior to our intervention. Notably, the optimal abstinence period for antibiotics before participating in a study assessing probiotics and gut microbiota can vary depending on the type of antibiotic used and the patient's metabolism. Therefore, the abstinence period is usually calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days (Armstrong, 2020). Hence, a one-week abstinence period preceding this study seemed reasonable to minimise the potential impact of residual antibiotics on gut microbiota and the study outcomes.

8.2 Sample Size

The sample size was determined using the minimum sample for a pilot clinical trial (Julious, 2005). A total of 61 patients were enrolled and randomised into the intervention (n = 30) and control (n = 31) groups, with an allocation ratio of 1:1.

8.3 Randomisation and Blinding

Patients were randomly allocated to either the intervention or control group using block randomisation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of [redacted]. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.

8.4 Intervention

The treatment was given to the patient upon their arrival. The intervention group received probiotics (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52 at a dose of 2×10^9 CFU) twice a day for eight weeks.

8.5 Outcomes

The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) at baseline and after the eight-week intervention. The secondary outcome was a change in serum serotonin levels after the eight weeks, measured using an enzyme-linked immunosorbent assay (ELISA).

8.6 Research Instruments

To assess the primary outcomes, the following instrument was used:

4) DASS-42

The DASS-42 is a 42-item questionnaire used to measure the severity of depression, anxiety, and stress in participants. The questionnaire consists of 42 assessment statements (supp 1) to assess depression (14 statements), anxiety (14 statements), and stress (14 statements). Participants were asked to score each statement as 0—*never*, 1—*sometimes*, 2—*often*, or 3—*very often*. Subscale scores were then summed to determine the depression, anxiety, and stress scale scores (supplementary file).

Meanwhile, to assess the secondary outcome, the following instrument was used:

2) Serotonin measurement

Serum serotonin levels were measured using an ELISA, following the manufacturer's protocol. Blood samples were collected at baseline and after the intervention, and results were read using a microplate reader (EL x 800).

8.7 Statistical Methods

This study used IBM SPSS Statistics 20 version for statistical analysis. Continuous variables (age, DASS-42 score, and serum serotonin level) were summarised using mean ± standard deviation, while categorical (nominal and ordinal) variables (gender, marital status, highest educational, jobs, history of psychiatric illness, history of psychiatric treatment, and duration of cancer diagnosis) were presented as frequencies and percentages.

For nominal variables (gender, marital status, and jobs), between group comparisons were conducted using chi-square test. Meanwhile, Mann-Whitney test was used for ordinal variables (highest education and duration since cancer diagnosis).

For continuous variables, Shapiro–Wilk test was used to assess the normality of data distribution. Between-group comparisons were conducted using independent t-test if the data was normally distributed and Mann–Whitney U test if the data was not normally distributed. Within-group comparisons were conducted using paired t-test if the data was normally distributed and using Wilcoxon signed rank test if the data was not normally distributed. A p-value < 0.05 was considered statistically significant.

An intention-to-treat (ITT) analysis was also performed to assess the effect of the intervention on several outcomes, including the total DASS score, it's subscales (depressions, anxiety, and stress), and serotonin level. All participants were analyzed based on control and

intervention groups regardless of whether they completed the study or not. The intended outcomes were the decrease in total DASS score and it's subscales, and increase in serotonin levels. The calculated metrics were control event rate (CER), experimental event rate (EER), absolute risk reduction (ARR), relative risk reduction (RRR), and number needed to treat (NNT).

8.8 Ethics

The study was registered at the Indonesian Clinical Research Registry (INA-CRR) with registration number 042024030706474KRGNHZ, and it was approved by the Health Research Ethics Committee of [redacted] (No. 1496/EC/KEPK-RSDK/2023). All participants provided written informed consent prior to participation. The trial was conducted according to the principles of the Declaration of Helsinki.

9. Results

3.1 Sample Characteristics

This study included 61 cancer patients who met the inclusion and exclusion criteria. The study sample was divided into two groups via randomisation, namely an intervention group and a control group. The baseline characteristics of the participants in the intervention (n=30) and control (n=31) groups are shown in supplementary file. There were no significant differences between the two groups for any of the baseline characteristics (age, cancer diagnosis, duration since cancer diagnosis, baseline DASS scores, and baseline serotonin level).

The primary reasons for dropout included unresponsiveness to contact attempts (45.7%), hospitalisation (20%), failure to attend control visits (20%), and death (11.4%) (supplementary file). Those who were unresponsive were participants whom the research team was unable to contact, despite repeated follow-up attempts. As a result, the intervention group comprised 13 subjects, so did the control group (figure 1).

Table 1 shows the characteristics of the study sample for each group. In this study sample, the mean ages in the intervention and control groups were 54.46 and 49.08 years, respectively. The number of male and female patients was approximately equal in the intervention and control groups. Most of the study sample was married (84.6%), had high school as their highest level of education (30.7%), and worked (65%). All samples had no history of psychiatric treatment or

previous psychiatric diagnoses. There were also no significant differences in their age, gender, marital status, educational level, employment status, and duration of cancer, either.

3.2 The Effect of Probiotics on Depression, Stress, and Anxiety

The results showed an insignificant decrease in depression ($p = 0.317$), anxiety ($p = 0.914$), and stress ($p = 0.581$) scores, while there was a significant decrease in total DASS-42 scores ($p = 0.001$), in the intervention group after receiving probiotics for eight weeks (Table 2). When comparing the control group with the intervention group, there were insignificant differences in depression, anxiety, and stress scores, but total DASS-42 scores significantly differed between the two groups ($p = 0.048$; Table 2).

3.3 The Effect of Probiotics on Serotonin

We found an increase in serotonin levels in the intervention group, but it was not statistically significant ($p = 0.382$; Table 3).

3.3 Intention-to-Treat (ITT) Analysis

As presented in supp 3, the CER, EER, ARR, RRR, and NNT provide early indicators of the intervention's potential impact. The results demonstrate a beneficial effect of probiotics in reducing overall psychological symptoms, as evidenced by increasing of total DASS-42 scores. However, the effect sized for the depression, anxiety, and stress subscales, as well as serotonin levels, were smaller and not statistically significant. The NNT value, despite exploratory, suggest that approximately nine patients would need to be treated with probiotics to achieve a reduction in total psychological symptoms in one patient.

10. Discussion

This pilot study investigated the preliminary effects of probiotic supplementation on psychological symptoms and serotonin levels in cancer patients undergoing chemotherapy. Cancer patients undergoing chemotherapy frequently experience significant psychological distress, including depression, anxiety, and stress, which adversely affects their quality of life (Ostovar et al., 2022). Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence the psychological symptoms of depression, anxiety, and stress via the gut–brain axis (Sabit et al., 2023). A systematic review also reported the beneficial effects of probiotic

supplementation measured using the Hamilton Depression Rating Scale in patients with psychiatric disorders (Amirani et al., 2020). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris et al., 2024; Potter et al., 2023; Ye et al., 2022; Zhang et al., 2023).

While probiotics have been shown to influence the gut-brain axis and improve psychological symptoms in other contexts, their effects on cancer patients undergoing chemotherapy are not well-established. Thus, this study provides preliminary insights into the feasibility and potential efficacy of probiotics in reducing psychological symptoms in this population.

The intention-to-treat (ITT) analysis was crucial in this pilot study to account for all randomized participants, including those who did not complete the study. The ITT approach provides a more realistic estimation of the treatment effect, especially in the study which has high dropout rate (Ahn & Kang, 2023).

The results indicate that the administration of probiotics over eight weeks caused a significant decrease in total DASS-42 scores ($p = 0.001$), suggesting an overall reduction in psychological distress. However, as a pilot study, these findings are exploratory and should be interpreted with caution. The observed number needed to treat (NNT) of 9 for total DASS-42 score reduction demonstrates that the intervention may have clinical relevance, but this needs confirmation in larger trials.

In terms of the specific DASS subscales (depression, anxiety, and stress), the decreases in the scores for each subscale were not statistically significant ($p > 0.005$). The most notable effect was seen in the stress subscale, with an NNT of 5, showing that probiotics may have a more positive effect on stress symptoms in cancer patients. However, the larger NNTs for the depression (8) and anxiety (17) subscales exhibit the need for further investigation with larger sample sizes to determine the true effect of probiotic supplementation on these specific psychological symptoms.

Chemotherapy, radiotherapy, and immunotherapy have toxic effects that can lead to changes in gut microbiota, a reduction in gut commensal bacteria, and inflammation of the gastrointestinal tract (Deleemans et al., 2019; Fernandes et al., 2024). Gut dysbiosis, a disruption in the gut microbiota, can lead to increased gut permeability, allowing toxins to enter the

bloodstream and activating pro-inflammatory cytokines (IL-6, II-1β, TNF-a, and C-reactive protein), while it may cause the hyperactivation of the hypothalamic–pituitary axis (HPA-axis) (Deleemans et al., 2019). These inflammatory conditions then lead to decreased levels of serotonin (5-HT) and BDNF (Deleemans et al., 2019). Both of these can, in turn, cause psychological and cognitive changes such as anxiety and depression, fatigue, memory impairment, and decision-making impairment (Lu et al., 2022; Maddern et al., 2023; Merkouris et al., 2024; Sabit et al., 2023).

The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker of stress. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.38$) and the NNT of 7 should be viewed as an exploratory finding. The control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, there are certain external factors that may have influenced these results. One such factor is the potential consumption of ondansetron. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels (Gupta et al., 2014), potentially explaining the more pronounced changes observed in the control group.

In addition, a diet that includes amino acids such as tryptophan, a precursor of serotonin, can contribute to variations in serotonin levels (Jenkins et al., 2016; Mohajeri et al., 2015) since tryptophan-rich foods can increase serotonin synthesis. However, dietary habits were not controlled for in this study, even though differences in diets among participants might have affected the results. Future studies should thus control for the medications and diets of their participants more rigorously.

An increase in serotonin is one of several mechanisms involved in the improvement of depression, anxiety, and stress symptoms from probiotic use. In addition to serotonin production, probiotics may reduce symptoms of depression and anxiety by decreasing stress-induced HPA responses, decreasing cortisol levels, increasing neurotransmitter synthesis (GABA, dopamine, noradrenaline, melatonin, histamine, and acetylcholine), stimulating the production of gut neuropeptides (glucagons like peptide-1 and tyrosine), improving the gut barrier, increasing BDNF

production, decreasing the release of pro-inflammatory cytokines, and increasing anti-inflammatory cytokines (Lu et al., 2022; Sabit et al., 2023; Zhang et al., 2023).

Overall, the gut–brain axis and serotonin production are influenced by numerous factors beyond just probiotic supplementation, including stress levels, diet, and physical activity (Lou et al., 2023; Madison & Kiecolt-Glaser, 2019; Mohajeri et al., 2015). The influence of these variables might have differed between the control and intervention groups, contributing to the outcomes seen in our study.

4.1 Limitations

This pilot study has several limitations that must be considered when interpreting the findings. First, although the intention-to-treat (ITT) analysis helped mitigate the impact of dropouts, the high dropout rate weakens the robustness of the findings. The high dropout rate was primarily driven by factors such as unresponsiveness, hospitalization, and death. Most of the dropout patients comprised individuals who were unresponsive to follow-up attempts (45.7%). Despite repeated attempts to contact them by telephone or messengers, participants could not be reached. Given that the comparison data of baseline characteristics between dropout and completer groups showed no significant differences, the risk of selection bias is minimized. However, the high dropout rate still poses a limitation to the statistical power, because the small sample size and homogeneity of the study population may limit the generalizability of the findings. Further larger-scale study should aim to implement strategies to improve participant retention, such as a more flexible follow-up options or using alternative contact methods, and include more diverse population to increase the generalizability of the findings.

A major concern raised by reviewers involves our choice of serum serotonin as a biomarker. While the direct impact of central nervous system (CNS) serotonin on the pathophysiology of depression, anxiety, and stress is well-recognised, the direct measurement of CNS serotonin is invasive and not feasible in a clinical trial setting. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may not accurately reflect CNS serotonin concentration. Although this was a limitation of our methodology, it is considered a feasible and ethical non-invasive sampling method for human subjects. Thus, many studies have already used this biomarker to investigate the systemic effects of probiotic supplementation (Jenkins et al., 2016; Merkouris et al., 2024; Potter et al., 2023; Yano et al., 2015). However, chemotherapy can

induce serotonin release from enterochromaffin cells in the gut (Cubeddu et al., 1995). This drug-induced serotonin release could have confounded the effects of probiotics. To mitigate this in future studies, alternative biomarkers should be utilized and other potential confounders such as chemotherapy type, anti-emetic use, and dietary factors should be controlled.

Another important limitation is that we did not apply any statistical correction to control for the potential inflation of Type I error because we conducted multiple comparisons. In future studies, statistical corrections should be applied when analyzing multiple outcomes.

Additionally, the duration of the intervention may have not been sufficient to detect meaningful changes in all outcomes. Studies have suggested that gut microbiota exist in a complex and dynamic ecosystem that may require longer probiotic interventions to undergo significant alterations (Ng et al., 2023). Another study also reported the beneficial effects of probiotic supplementation on symptoms of depression after 12 weeks among patients under methadone maintenance treatment programmes (Molavi et al., 2022). Thus, extending the duration of probiotic supplementation in future trial could determine whether longer-term treatment will give more pronounced effects.

Finally, we did not control for other external factors, such as physical activity, dietary habits, medication use, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Controlling for these variables in future research is crucial to better understanding the direct effects of probiotics on psychological symptoms in cancer patients.

11. Conclusions

This pilot study provides preliminary evidence that eight weeks of probiotic supplementation may have a potential role in reducing overall psychological symptoms in cancer patients undergoing chemotherapy, as shown by changes in total DASS-42 scores. However, the results should be interpreted carefully due to the small sample size, high dropout rate, and limitations associated with other factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Future larger trials with more rigorous controls and longer intervention periods are needed to confirm these preliminary findings and to further explore

the therapeutic potential of probiotics on psychological symptoms in cancer patients undergoing chemotherapy.

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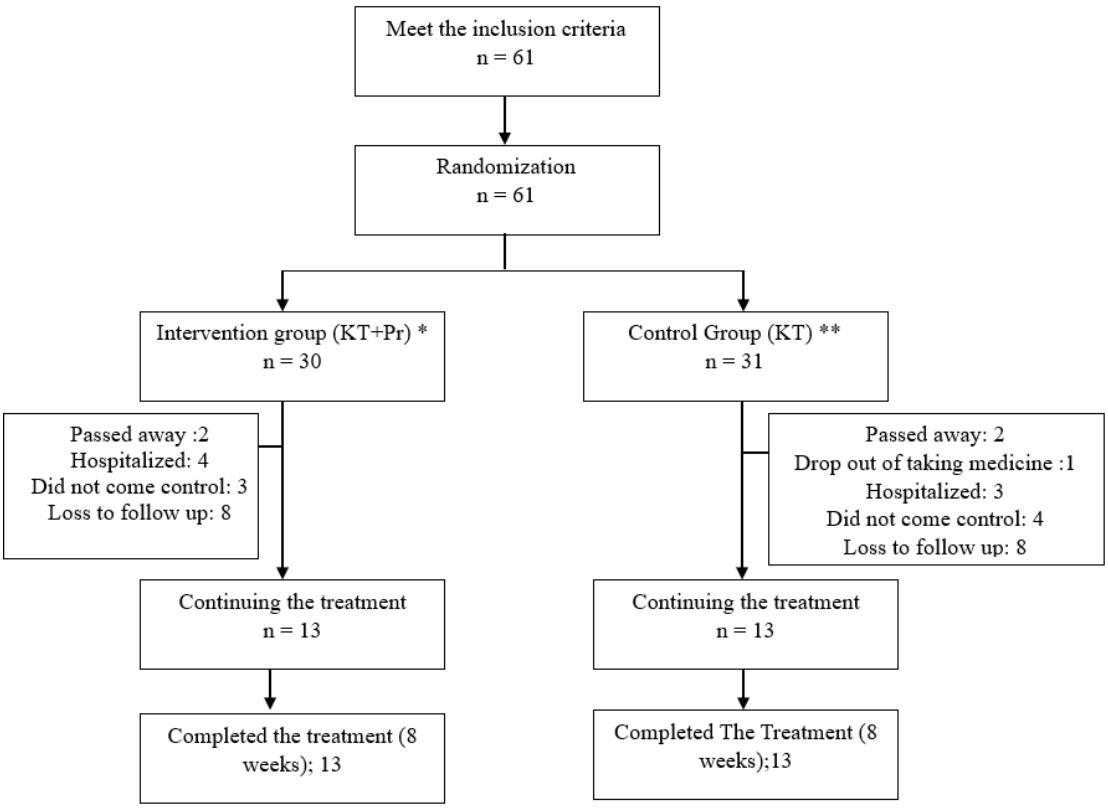

Figure 1. CONSORT diagram. This study included 61 cancer patients, randomised into an intervention group and a control group.

Description:

*KT + Pr = Chemotherapy + probiotics

**KT = Chemotherapy + placebo

Table 1. Characteristics of the research sample

Variables	**Intervention (*n* = 13)**	**Control (*n* = 13)**	***p*-value***
Age			
Mean ± SD	54 ± 7.88	49 ± 17.06	0.08§
Median (min–max)	54 (43–68)	54 (23–76)	
	Frequency (%)		
Variables	**Intervention (*n* = 13)**	**Control (*n* = 13)**	***p*-value**

Gender			
Male	7 (53.8%)	6 (46.2%)	1.00[¥]
Female	6 (46.2%)	7 (53.8%)	
Marital status			
Not married	1 (7.7%)	2 (15.4%)	0.70[¥]
Married	11 (84.6%)	11 (84.6%)	
Divorced	1 (7.7%)	0 (0%)	
Highest education			
Elementary school	1 (7.7%)	1 (7.7%)	0.71[‡]
Junior high school	2 (15.4%)	4 (30.8%)	
Senior high school	5 (38.5%)	3 (23.1%)	
Bachelor	4 (30.8%)	4 (30.8%)	
Did not attend school	1 (7.7%)	1 (7.7%)	
Jobs			
Working	8 (61.5%)	9 (69.2%)	0.50[¥]
Not working	5 (38.5%)	4 (30.8%)	
History of psychiatric treatment (including benzodiazepine)			
Yes	0	0	.[e]
No	13 (50%)	13 (50%)	
Psychiatric diagnosis			
Yes	0	0	.[e]
No	13 (50%)	13 (50%)	
Duration of psychiatric treatment			
Yes	0	0	.[e]
No	13 (50%)	13 (50%)	
Duration since cancer diagnosis			
3–6 months	2 (15.4%)	4 (30.8%)	0.52[‡]
6 months–1 year	5 (38.5%)	4 (30.8%)	

1–5 years	5 (38.5%)	4 (30.8%)
> 5 years	1 (7.7%)	1 (7.7%)

‡Mann–Whitney; §independent t; ¥chi-square; ᵉnot measurable because $n = 0$, *$p < 0.05$ was considered statistically significant

Table 2. Comparison of depression scores for the intervention and control groups before and after the intervention

DASS-42	Group		*p*
	Intervention	**Control**	
Pre-intervention	19.00 ± 7.10	13.69 ± 7.06	0.07§ (95% CI -0.42, 11.04)
Post-intervention	17.38 ± 6.48	11.15 ± 6.83	0.048‡*
p	0.001¶* (95% CI 0.78, 2.45)	0.002†*	0.21§
Depression			
Pre-intervention	5.69 ± 3.20	6.31 ± 3.77	0.658§ (95% CI -3.4, 2.2)
Post-intervention	5.46 ± 3.05	4.69 ± 2.78	0.508§ (95% CI -1.6, 3.1)
p	0.32†	0.01†*	0.06‡
Anxiety			
Pre-intervention	5.23 ± 3.86	5.15 ± 8.16	0.188‡
Post-intervention	5.46 ± 4.05	3.54 ± 4.82	0.055‡
p	0.91†	0.02†*	0.08‡
Stress			
Pre-intervention	6.15 ± 3.02	9.15 ± 5.54	0.099§ (95% CI -6.6, 0.6)
Post-intervention	6.46 ± 2.33	6.92 ± 4.27	0.735§ (95% CI -3.2, 2.3)
p	0.58†	0.007†*	0.003‡

* Statistically significant ($p < 0.05$); ‡ Mann–Whitney; § independent t; ¶ paired t; † Wilcoxon

Table 3. Comparison of serotonin levels between the intervention and control groups before and after the intervention

Serotonin	Group		*p*
	Intervention	Control	
Pre-intervention	98.85 ± 125.22	145.77 ± 199.78	0.80‡
Post-intervention	104.15 ± 195.69	161.38 ± 175.37	0.01‡*
p	0.38†	0.09†	
Difference	5.31 ± 77.48	15.62 ± 66.20	0.048‡*

*Statistically significant ($p < 0.05$); ‡ Mann–Whitney; † Wilcoxon

Paper Accepted (14-10-2024)

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Probiotic Supplementation for Reducing Psychological Symptoms in Cancer Patients on Chemotherapy: a Pilot Trial

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Probiotic supplementation for reducing psychological symptoms in cancer patients on chemotherapy: A pilot trial

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ABSTRACT

Background: Psychological disorders, including depression, anxiety, and stress, are prevalent among cancer patients undergoing chemotherapy. Probiotics have been investigated as a potential supplement to modulate the gut–brain axis and improve psychological symptoms, possibly through mechanisms such as serotonin regulation. However, studies on the effects of probiotics on psychological symptoms in chemotherapy patients are limited.
Methods: This randomised, double-blinded, placebo-controlled pilot trial was conducted at the outpatient clinic of dr. Kariadi Hospital, Semarang, in 2023. Sixty-one cancer patients undergoing chemotherapy were enrolled and randomised into an intervention ($n = 30$) and control ($n = 31$) group. The intervention group received probiotics (*Lactobacillus rhamnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) twice daily for eight weeks. The primary outcomes were changes in depression, anxiety, and stress levels measured by the Depression, Anxiety, and Stress Scale 42 (DASS-42). The secondary outcome was serum serotonin levels.
Results: The intervention group showed a significant decrease in total DASS-42 scores ($p = 0.001$), indicating an overall reduction in psychological distress. However, changes in the scores of the subscales (depression, anxiety, and stress) were not statistically significant ($p > 0.05$). Serum serotonin levels increased in the intervention group, but this was not statistically significant ($p = 0.38$). The findings should be interpreted cautiously due to small sample size and potential confounding factors.
Conclusion: This pilot study suggests that eight weeks of probiotic supplementation may reduce overall psychological symptoms in cancer patients undergoing chemotherapy. Larger trials with rigorous controls and longer interventions are needed to confirm these preliminary findings.

1. Introduction

Psychological disorders are becoming increasingly prevalent among patients diagnosed with cancer, with conditions such as delirium, depression, adjustment disorders, anxiety, sexual dysfunction, and sleep disorders affecting 30 %–40 % of this population (Mastan et al., 2024; Ostovar et al., 2022). The incidence of psychiatric disorders is even higher among those at advanced cancer stages. Anxiety was more common (varying from 7 % to 88 %) than depression (ranging from 3 % to 65.5 %) among cancer patients with diverse forms residing in different Southeast Asian nations (Ostovar et al., 2022). Additionally, a study in one Indonesian hospital reported that 23 % of patients undergoing chemotherapy experienced depression, 40 % suffered from anxiety, and 21 % had stress (Mastan et al., 2024). Despite the significant impact of these conditions on patients' quality of life, psychiatric disorders in cancer patients are often underdiagnosed and inadequately treated, leading to further deterioration in their overall wellbeing (Mastan et al., 2024; Ostovar et al., 2022; S et al., 2018).

Chemotherapy has been shown to disrupt the balance of gut microbiota, a condition known as gut dysbiosis (Deleemans et al., 2019). This disruption can lead to a reduction in the diversity and number of commensal bacteria, which, in turn, can negatively affect mood and

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cognitive function (Deleemans et al., 2019; Maddern et al., 2023). Research has demonstrated that changes in microbiota composition can influence the development of the psychological symptoms of conditions including depression and anxiety (Deleemans et al., 2019; Maddern et al., 2023). Animal studies have further demonstrated that gut microbiota play a critical role in regulating the pathway associated with depression (Deleemans et al., 2019), suggesting that gut microbiota may be a promising therapeutic target for psychological disorders among cancer patients.

Since psychological disorders are linked to low serotonin levels—a condition that can be exacerbated by gut dysbiosis—targeting the gut microbiota through oral probiotics presents a promising therapeutic approach (Merkouris et al., 2024; Zhang et al., 2023). Probiotics have traditionally been used to mitigate the gastrointestinal side-effects of chemotherapy, such as nausea and vomiting (Vivarelli et al., 2019). Recent studies have suggested that probiotics may additionally improve psychological wellbeing by influencing neurotransmitter pathways, including serotonin pathways, which are closely associated with mood regulation (Merkouris et al., 2024; Zhang et al., 2023). Specifically, probiotics containing *Lactobacillus rhamnosus* and *Lactobacillus helveticus* have been shown to reduce symptoms of depression, enhance cognitive function, and balance key neurochemicals, including serotonin, epinephrine, and brain-derived neurotrophic factor (BDNF), in animal studies (Deleemans et al., 2019; Ye et al., 2022).

Despite these promising findings, existing researches have predominantly focused on the effects of probiotics on the physical side-effects of cancer and chemotherapy, with limited studies specifically evaluating their efficacy in managing psychological conditions in chemotherapy patients. In addition, the influence of the combination of probiotics used in this study (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52) on psychological disorders in a human sample has never been studied. Therefore, this pilot study aims to assess the feasibility and acceptability of probiotic supplementation to reduce psychological symptoms in cancer patients undergoing chemotherapy. The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) and the secondary outcome was a change in serum serotonin levels.

2. Methods

This study was a randomised, double-blinded, placebo-controlled pilot trial conducted in the outpatient clinic of Kasuari Ward of Dr. Kariadi Hospital, Semarang in 2023. The trial was designed to assess the effect of probiotic supplementation on depression, anxiety, and stress in cancer patients undergoing chemotherapy, using serum serotonin levels as a biomarker.

2.1. Participants

Participants included cancer patients who were undergoing chemotherapy in Dr Kariadi Hospital. The only inclusion criterion was that patients had to be aged between 18 and 76 years. Exclusion criteria were patients who smoked or had used antibiotics during the week prior to our intervention. Notably, the optimal abstinence period for antibiotics before participating in a study assessing probiotics and gut microbiota can vary depending on the type of antibiotic used and the patient's metabolism. Therefore, the abstinence period is usually calculated based on the drug's half-life. Most antibiotics have a half-life ranging from a few hours to a few days (Armstrong, 2020). Hence, a one-week abstinence period preceding this study seemed reasonable to minimise the potential impact of residual antibiotics on gut microbiota and the study outcomes.

2.2. Sample size

The sample size was determined using the minimum sample for a pilot clinical trial (Julious, 2005). A total of 61 patients were enrolled and randomised into the intervention ($n = 30$) and control ($n = 31$) groups, with an allocation ratio of 1:1.

2.3. Randomisation and blinding

Patients were randomly allocated to either the intervention or control group using block randomisation, with a block size of four (ABAB, BABA, AABB, BBAA) and an allocation ratio of 1:1. The block order was stored in a sealed envelope and was only opened after the study was completed. The treatment code was also included in the envelope and was numbered according to the block order. Blinding was maintained by ensuring that the probiotic and placebo capsules were identical in appearance (colour, size, and shape), packaging, and administration. The placebo capsules were manufactured by the pharmaceutical laboratory of Medical Faculty, Diponegoro University. The placebo capsule contained the same additional substances as the probiotic capsule, namely maltodextrin, magnesium stearate, and ascorbic acid. These substances did not interfere with the research results. Both patients and investigators were blinded to the group assignments; only the pharmacist knew the group assignments.

2.4. Intervention

The treatment was given to the patient upon their arrival. The intervention group received probiotics (*Lactobacillus rhomnosus* Rosell-11 and *Lactobacillus helveticus* Rosell-52 at a dose of 2×10^9 CFU) twice a day for eight weeks.

2.5. Outcomes

The primary outcome was a change in depression, anxiety, and stress levels, measured using the Depression, Anxiety, and Stress Scale 42 (DASS-42) at baseline and after the eight-week intervention. The secondary outcome was a change in serum serotonin levels after the eight weeks, measured using an enzyme-linked immunosorbent assay (ELISA).

2.6. Research instruments

To assess the primary outcomes, the following instrument was used:

1) DASS-42
The DASS-42 is a 42-item questionnaire used to measure the severity of depression, anxiety, and stress in participants. The questionnaire consists of 42 assessment statements (supplementary file) to assess depression (14 statements), anxiety (14 statements), and stress (14 statements). Participants were asked to score each statement as 0—*never*, 1—*sometimes*, 2—*often*, or 3—*very often*. Subscale scores were then summed to determine the depression, anxiety, and stress scale scores (supplementary file).

Meanwhile, to assess the secondary outcome, the following instrument was used:

2) Serotonin measurement

Serum serotonin levels were measured using an ELISA, following the manufacturer's protocol. Blood samples were collected at baseline and after the intervention, and results were read using a microplate reader (EL x 800).

2.7. Statistical methods

This study used SPSS version 20 for statistical analysis. Continuous variables (age, DASS-42 score, and serum serotonin level) were

summarised using mean ± standard deviation, while categorical (nominal and ordinal) variables (gender, marital status, highest educational, jobs, history of psychiatric illness, history of psychiatric treatment, and duration of cancer diagnosis) were presented as frequencies and percentages.

For nominal variables (gender, marital status, and jobs), between group comparisons were conducted using chi-square test. Meanwhile, Mann-Whitney test was used for ordinal variables (highest education and duration since cancer diagnosis).

For continuous variables, Shapiro–Wilk test was used to assess the normality of data distribution. Between-group comparisons were conducted using independent *t*-test if the data was normally distributed and Mann–Whitney U test if the data was not normally distributed. Within-group comparisons were conducted using paired *t*-test if the data was normally distributed and using Wilcoxon signed rank test if the data was not normally distributed. A p-value < 0.05 was considered statistically significant.

An intention-to-treat (ITT) analysis was also performed to assess the effect of the intervention on several outcomes, including the total DASS score, it's subscales (depressions, anxiety, and stress), and serotonin level. All participants were analyzed based on control and intervention groups regardless of whether they completed the study or not. The intended outcomes were the decrease in total DASS score and it's subscales, and increase in serotonin levels. The calculated metrics were control event rate (CER), experimental event rate (EER), absolute risk reduction (ARR), relative risk reduction (RRR), and number needed to treat (NNT).

2.8. Ethics

The study was registered at the Indonesian Clinical Research Registry (INA-CRR) with registration number 042024030706474KRGNHZ, and it was approved by the Health Research Ethics Committee of Dr. Kariadi Hospital, Semarang (No. 1496/EC/KEPK-RSDK/2023). All participants provided written informed consent prior to participation. The trial was conducted according to the principles of the Declaration of Helsinki.

3. Results

3.1. Sample characteristics

This study included 61 cancer patients who met the inclusion and exclusion criteria. The study sample was divided into two groups via randomisation, namely an intervention group and a control group. The baseline characteristics of the participants in the intervention ($n = 30$) and control ($n = 31$) groups are shown in supplementary file. There were no significant differences between the two groups for any of the baseline characteristics (age, cancer diagnosis, duration since cancer diagnosis, baseline DASS scores, and baseline serotonin level).

The primary reasons for dropout included unresponsiveness to contact attempts (45.7 %), hospitalisation (20 %), failure to attend control visits (20 %), and death (11.4 %) (supplementary file). Those who were unresponsive were participants whom the research team was unable to contact, despite repeated follow-up attempts. As a result, the intervention group comprised 13 subjects, so did the control group (Fig. 1).

Table 1 shows the characteristics of the study sample for each group.

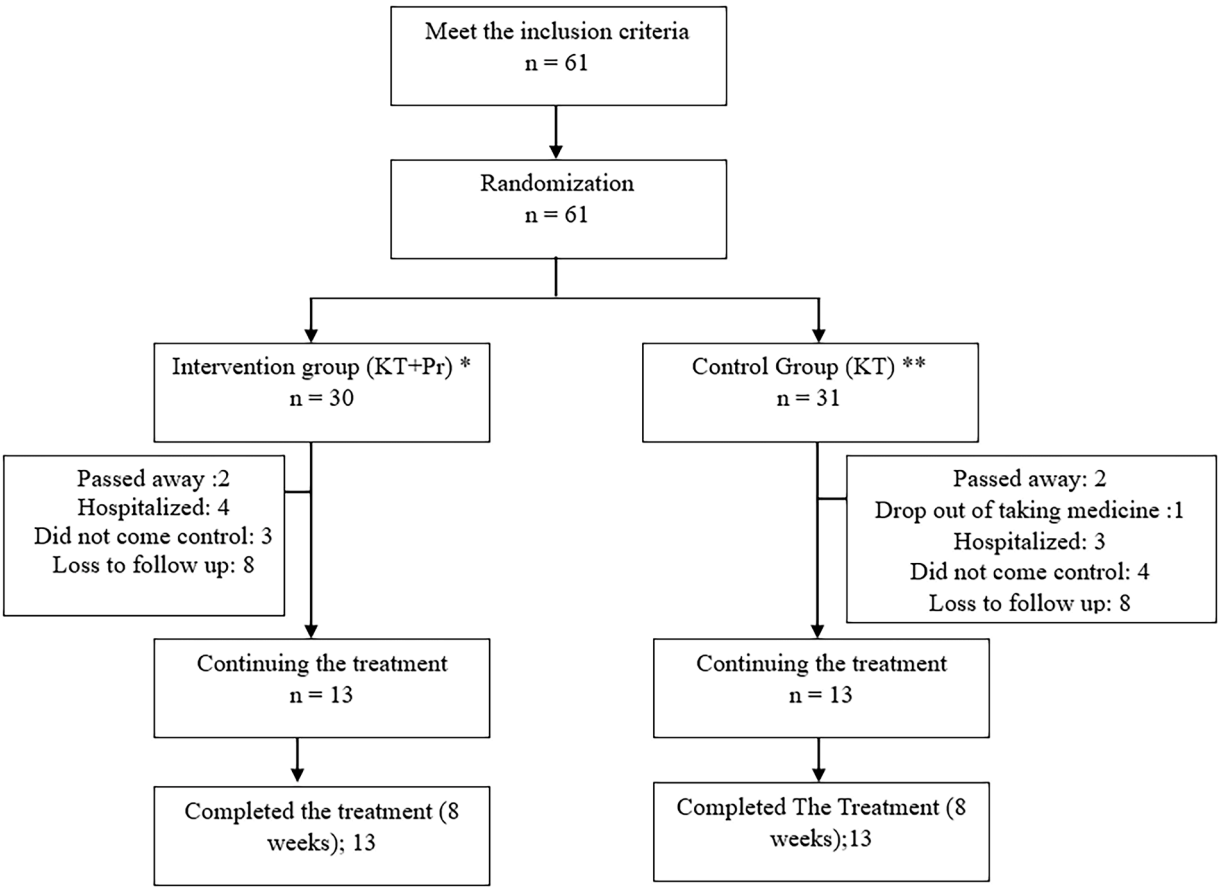

Fig. 1. CONSORT diagram. This study included 61 cancer patients, randomised into an intervention group and a control group.
Description:
*KT + Pr = Chemotherapy + probiotics
**KT = Chemotherapy + placebo.

Table 1
Characteristics of the research sample.

Variables	Intervention (n = 13)	Control (n = 13)	p-value [*]
Age			
Mean ± SD	54 ± 7.88	49 ± 17.06	0.08[§]
Median (min–max)	54 (43–68)	54 (23–76)	
Variables	**Frequency (%) Intervention (n = 13)**	**Control (n = 13)**	***p*-value**
Gender			
Male	7 (53.8 %)	6 (46.2 %)	1.00[¥]
Female	6 (46.2 %)	7 (53.8 %)	
Marital status			
Not married	1 (7.7 %)	2 (15.4 %)	0.70[¥]
Married	11 (84.6 %)	11 (84.6 %)	
Divorced	1 (7.7 %)	0 (0 %)	
Highest education			
Elementary school	1 (7.7 %)	1 (7.7 %)	0.71[‡]
Junior high school	2 (15.4 %)	4 (30.8 %)	
Senior high school	5 (38.5 %)	3 (23.1 %)	
Bachelor	4 (30.8 %)	4 (30.8 %)	
Did not attend school	1 (7.7 %)	1 (7.7 %)	
Jobs			
Working	8 (61.5 %)	9 (69.2 %)	0.50[¥]
Not working	5 (38.5 %)	4 (30.8 %)	
History of psychiatric treatment (including benzodiazepine)			
Yes	0	0	.[e]
No	13 (50 %)	13 (50 %)	
Psychiatric diagnosis			
Yes	0	0	.[e]
No	13 (50 %)	13 (50 %)	
Duration of psychiatric treatment			
Yes	0	0	.[e]
No	13 (50 %)	13 (50 %)	
Duration since cancer diagnosis			
3–6 months	2 (15.4 %)	4 (30.8 %)	0.52[‡]
6 months–1 year	5 (38.5 %)	4 (30.8 %)	
1–5 years	5 (38.5 %)	4 (30.8 %)	
> 5 years	1 (7.7 %)	1 (7.7 %)	

[‡] Mann–Whitney;.
[§] independent t;.
[¥] chi-square;.
[e] not measurable because $n = 0$,.
[*] $p < 0.05$ was considered statistically significant.

In this study sample, the mean ages in the intervention and control groups were 54 and 49 years, respectively. The number of male and female patients was approximately equal in the intervention and control groups. Most of the study sample was married (84.6 %), had high school as their highest level of education (30.7 %), and worked (65 %). All samples had no history of psychiatric treatment or previous psychiatric diagnoses. There were also no significant differences in their age, gender, marital status, educational level, employment status, and duration of cancer, either.

3.2. The effect of probiotics on depression, stress, and anxiety

The results showed an insignificant decrease in depression (p = 0.32), anxiety ($p = 0.91$), and stress ($p = 0.58$) scores, while there was a significant decrease in total DASS-42 scores ($p = 0.001$), in the intervention group after receiving probiotics for eight weeks (Table 2). When comparing the control group with the intervention group, there were insignificant differences in depression, anxiety, and stress scores, but total DASS-42 scores significantly differed between the two groups (p = 0.048; Table 2).

3.3. The effect of probiotics on serotonin

We found an increase in serotonin levels in the intervention group, but it was not statistically significant ($p = 0.38$; Table 3).

Table 2
Comparison of depression scores for the intervention and control groups before and after the intervention.

DASS-42	Group		p
	Intervention	Control	
Pre-intervention	19.00 ± 7.10	13.69 ± 7.06	0.07[§] (95 % CI −0.42, 11.04)
Post-intervention	17.38 ± 6.48	11.15 ± 6.83	0.048[‡,*]
P	0.001[¶,*] (95 % CI 0.78, 2.45)	0.002[†,*]	0.21[§]
Depression			
Pre-intervention	5.69 ± 3.20	6.31 ± 3.77	0.658[§] (95 % CI −3.4, 2.2)
Post-intervention	5.46 ± 3.05	4.69 ± 2.78	0.508[§] (95 % CI −1.6, 3.1)
P	0.32[†]	0.01[†,*]	0.06[‡]
Anxiety			
Pre-intervention	5.23 ± 3.86	5.15 ± 8.16	0.188[‡]
Post-intervention	5.46 ± 4.05	3.54 ± 4.82	0.055[‡]
P	0.91[†]	0.02[†,*]	0.08[‡]
Stress			
Pre-intervention	6.15 ± 3.02	9.15 ± 5.54	0.099[§] (95 % CI −6.6, 0.6)
Post-intervention	6.46 ± 2.33	6.92 ± 4.27	0.735[§] (95 % CI −3.2, 2.3)
P	0.58[†]	0.007[†,*]	0.003[‡]

[*] Statistically significant ($p < 0.05$);.
[‡] Mann–Whitney;.
[§] independent t;.
[¶] paired t;.
[†] Wilcoxon.

3.3. Intention-to-Treat (ITT) analysis

As presented in supplementary file, the CER, EER, ARR, RRR, and NNT provide early indicators of the intervention's potential impact. The results demonstrate a beneficial effect of probiotics in reducing overall psychological symptoms, as evidenced by increasing of total DASS-42 scores. However, the effect sized for the depression, anxiety, and stress subscales, as well as serotonin levels, were smaller and not statistically significant. The NNT value, despite exploratory, suggest that approximately nine patients would need to be treated with probiotics to achieve a reduction in total psychological symptoms in one patient.

4. Discussion

This pilot study investigated the preliminary effects of probiotic supplementation on psychological symptoms and serotonin levels in cancer patients undergoing chemotherapy. Cancer patients undergoing chemotherapy frequently experience significant psychological distress, including depression, anxiety, and stress, which adversely affects their

Table 3
Comparison of serotonin levels between the intervention and control groups before and after the intervention.

Serotonin	Group		p
	Intervention	Control	
Pre-intervention	98.85 ± 125.22	145.77 ± 199.78	0.80[‡]
Post-intervention	104.15 ± 195.69	161.38 ± 175.37	0.01[‡,*]
P	0.38[†]	0.09[†]	
Difference	5.31 ± 77.48	15.62 ± 66.20	0.048[‡,*]

[*] Statistically significant ($p < 0.05$);.
[‡] Mann–Whitney;.
[†] Wilcoxon.

quality of life (Ostovar et al., 2022). Probiotic supplementation has been explored for its potential to modulate gut microbiota and influence the psychological symptoms of depression, anxiety, and stress via the gut–brain axis (Sabit et al., 2023). A systematic review also reported the beneficial effects of probiotic supplementation measured using the Hamilton Depression Rating Scale in patients with psychiatric disorders (Amirani et al., 2020). However, the effects of probiotics on these outcomes have shown variability across studies (Merkouris et al., 2024; Potter et al., 2023; Ye et al., 2022; Zhang et al., 2023).

While probiotics have been shown to influence the gut-brain axis and improve psychological symptoms in other contexts, their effects on cancer patients undergoing chemotherapy are not well-established. Thus, this study provides preliminary insights into the feasibility and potential efficacy of probiotics in reducing psychological symptoms in this population.

The intention-to-treat (ITT) analysis was crucial in this pilot study to account for all randomized participants, including those who did not complete the study. The ITT approach provides a more realistic estimation of the treatment effect, especially in the study which has high dropout rate (Ahn & Kang, 2023).

The results indicate that the administration of probiotics over eight weeks caused a significant decrease in total DASS-42 scores ($p = 0.001$), suggesting an overall reduction in psychological distress. However, as a pilot study, these findings are exploratory and should be interpreted with caution. The observed number needed to treat (NNT) of 9 for total DASS-42 score reduction demonstrates that the intervention may have clinical relevance, but this needs confirmation in larger trials.

In terms of the specific DASS subscales (depression, anxiety, and stress), the decreases in the scores for each subscale were not statistically significant ($p > 0.05$). The most notable effect was seen in the stress subscale, with an NNT of 5, showing that probiotics may have a more positive effect on stress symptoms in cancer patients. However, the larger NNTs for the depression (8) and anxiety (17) subscales exhibit the need for further investigation with larger sample sizes to determine the true effect of probiotic supplementation on these specific psychological symptoms.

Chemotherapy, radiotherapy, and immunotherapy have toxic effects that can lead to changes in gut microbiota, a reduction in gut commensal bacteria, and inflammation of the gastrointestinal tract (Deleemans et al., 2019; Fernandes et al., 2024). Gut dysbiosis, a disruption in the gut microbiota, can lead to increased gut permeability, allowing toxins to enter the bloodstream and activating pro-inflammatory cytokines (IL-6, II-1β, TNF-a, and C-reactive protein), while it may cause the hyperactivation of the hypothalamic–pituitary axis (HPA-axis) (Deleemans et al., 2019). These inflammatory conditions then lead to decreased levels of serotonin (5-HT) and BDNF (Deleemans et al., 2019). Both of these can, in turn, cause psychological and cognitive changes such as anxiety and depression, fatigue, memory impairment, and decision-making impairment (Lu et al., 2022; Maddern et al., 2023; Merkouris et al., 2024; Sabit et al., 2023).

The mechanism of psychological disturbance discussed in our study is related to chemotherapy-induced gut dysbiosis, with serum serotonin as a biomarker. In this study, while the intervention group receiving probiotic supplementation showed an increase in serum serotonin levels, this change was not statistically significant ($p = 0.38$) and the NNT of 7 should be viewed as an exploratory finding. The control group, which did not receive probiotics, demonstrated more pronounced changes in serum serotonin levels. Hence, there are certain external factors that may have influenced these results. One such factor is the potential consumption of ondansetron. Ondansetron, a commonly used antiemetic in chemotherapy patients, is known to influence serotonin levels by blocking serotonin 5-HT3 receptors (Gupta et al., 2014). This pharmacological action can lead to fluctuations in circulating serotonin levels (Gupta et al., 2014), potentially explaining the more pronounced changes observed in the control group.

In addition, a diet that includes amino acids such as tryptophan, a precursor of serotonin, can contribute to variations in serotonin levels (Jenkins et al., 2016; Mohajeri et al., 2015) since tryptophan-rich foods can increase serotonin synthesis. However, dietary habits were not controlled for in this study, even though differences in diets among participants might have affected the results. Future studies should thus control for the medications and diets of their participants more rigorously.

An increase in serotonin is one of several mechanisms involved in the improvement of depression, anxiety, and stress symptoms from probiotic use. In addition to serotonin production, probiotics may reduce symptoms of depression and anxiety by decreasing stress-induced HPA responses, decreasing cortisol levels, increasing neurotransmitter synthesis (GABA, dopamine, noradrenaline, melatonin, histamine, and acetylcholine), stimulating the production of gut neuropeptides (glucagons like peptide-1 and tyrosine), improving the gut barrier, increasing BDNF production, decreasing the release of pro-inflammatory cytokines, and increasing anti-inflammatory cytokines (Lu et al., 2022; Sabit et al., 2023; Zhang et al., 2023).

Overall, the gut–brain axis and serotonin production are influenced by numerous factors beyond just probiotic supplementation, including stress levels, diet, and physical activity (Lou et al., 2023; Madison & Kiecolt-Glaser, 2019; Mohajeri et al., 2015). The influence of these variables might have differed between the control and intervention groups, contributing to the outcomes seen in our study.

4.1. Limitations

This pilot study has several limitations that must be considered when interpreting the findings. First, although the intention-to-treat (ITT) analysis helped mitigate the impact of dropouts, the high dropout rate weakens the robustness of the findings. The high dropout rate was primarily driven by factors such as unresponsiveness, hospitalization, and death. Most of the dropout patients comprised individuals who were unresponsive to follow-up attempts (45.7 %). Despite repeated attempts to contact them by telephone or messengers, participants could not be reached. Given that the comparison data of baseline characteristics between dropout and completer groups showed no significant differences, the risk of selection bias is minimized. However, the high dropout rate still poses a limitation to the statistical power, because the small sample size and homogeneity of the study population may limit the generalizability of the findings. Further larger-scale study should aim to implement strategies to improve participant retention, such as a more flexible follow-up options or using alternative contact methods, and include more diverse population to increase the generalizability of the findings.

Another major concern is the use of serum serotonin as a biomarker. While the direct impact of central nervous system (CNS) serotonin on the pathophysiology of depression, anxiety, and stress is well-recognised, the direct measurement of CNS serotonin is invasive and not feasible in a clinical trial setting. We acknowledge that serum serotonin can be influenced by various peripheral factors, and it may not accurately reflect CNS serotonin concentration. Although this was a limitation of our methodology, it is considered a feasible and ethical non-invasive sampling method for human subjects. Thus, many studies have already used this biomarker to investigate the systemic effects of probiotic supplementation (Jenkins et al., 2016; Merkouris et al., 2024; Potter et al., 2023; Yano et al., 2015). However, chemotherapy can induce serotonin release from enterochromaffin cells in the gut (Cubeddu et al., 1995). This drug-induced serotonin release could have confounded the effects of probiotics. To mitigate this in future studies, alternative biomarkers should be utilized and other potential confounders such as chemotherapy type, anti-emetic use, and dietary factors should be controlled.

Another important limitation is that we did not apply any statistical correction to control for the potential inflation of Type I error because we conducted multiple comparisons. In future studies, statistical corrections should be applied when analyzing multiple outcomes.

Additionally, the duration of the intervention may have not been sufficient to detect meaningful changes in all outcomes. Studies have suggested that gut microbiota exist in a complex and dynamic ecosystem that may require longer probiotic interventions to undergo significant alterations (Ng et al., 2023). Another study also reported the beneficial effects of probiotic supplementation on symptoms of depression after 12 weeks among patients under methadone maintenance treatment programmes (Molavi et al., 2022). Thus, extending the duration of probiotic supplementation in future trial could determine whether longer-term treatment will give more pronounced effects.

Finally, we did not control for other external factors, such as physical activity, dietary habits, medication use, and other lifestyle factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Controlling for these variables in future research is crucial to better understanding the direct effects of probiotics on psychological symptoms in cancer patients.

5. Conclusions

This pilot study provides preliminary evidence that eight weeks of probiotic supplementation may have a potential role in reducing overall psychological symptoms in cancer patients undergoing chemotherapy, as shown by changes in total DASS-42 scores. However, the results should be interpreted carefully due to the small sample size, high dropout rate, and limitations associated with other factors that may influence the gut microbiota, serotonin levels, and psychological disorders of cancer patients. Future larger trials with more rigorous controls and longer intervention periods are needed to confirm these preliminary findings and to further explore the therapeutic potential of probiotics on psychological symptoms in cancer patients undergoing chemotherapy.

CRediT authorship contribution statement

Alifiati Fitrikasari: Conceptualization, Methodology, Data curation, Writing – original draft. **Innawati Jusup:** Methodology, Validation. **Titis Hadiati:** Formal analysis, Project administration. **Widodo Sarjana:** Data curation. **Salytha Ivana Ardiningrum:** Writing – review & editing. **Cindy Kurniawati Chandra:** Writing – review & editing. **Damai Santosa:** Writing – original draft, Resources.

Declaration of competing interest

We have no known conflicts of interest to disclose.

Declaration of generative AI in scientific writing

During the preparation of this work, the authors used ChatGPT 4.0 to improve the grammar and clarity of the manuscript, as English is not the authors' first language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Supplementary materials

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