SNOWDEN CO.,LTD.
3-7-16, Iwamoto-cho, Chiyoda-ku, Tokyo TEL.03-3866-2828

ThreeLac Safety Data Report

Notification: ThreeLac and FiveLac are exclusively distributed by The GHT Companies, in representation of the Founding Scientist, Snowden Co., Ltd. Any and all inquiries should be directed to The GHT Companies, Customer Service, at 1-800-305-5710.

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Introduction

Over the past few years, our companies have been urging Snowden Co., Ltd., the Founding Scientist of both ThreeLac and FiveLac, to generate a series of documents that would provide supportive safety data and test results to serve as a reliable resource for our Distribution Partners, as well as the End-User/Consumer, in regard to the continued nontoxic use of our very effective probiotic blends.

The Founding Scientist presents a historical approach to the development of the products, which would include the careful selection of the bacteria strains. There was extraordinary focus placed on the selection of the strains for both effectiveness and safety. Additionally, the selection process was carefully orchestrated to develop the best combination of ingredients that would survive the gastric environment and deliver the probiotic blend to the targeted area, the intestinal tract. This particular report does not focus as much on this factor, as it does on the work effort to produce a safe product for human consumption.

There has been much written about the use of the *Enterococcus faecalis* strain, due to its reputation for possible pathogenic results. In fact, the selected strain used in both of the products is *Enterococcus faecalis* F-100, which is a sub-strain of the well-documented *Enterococcus faecalis*. In the initial document of this report, the Founding Scientist outlines the selection process, the reasons for the choices, and they have clearly stated their position on the safety of this strain, along with all of the strains selected for ThreeLac and FiveLac. We believe this to be a powerful and informative position taken by Snowden Co., Ltd.

In the Table of Contents, listed are a series of five documents, which include a direct Statement on Safety, an In-Vitro Study for Determining Safety Characteristics, Oral Toxicity Studies by a third party testing facility, a Study on the Inhibition of Biofilm in Candida albicans, and finally, a small Human Double Blind Study.

Development and implementation of a larger human study in regard to continued research focused on the effectiveness of ThreeLac for Candidiasis will be conducted in the future.

Finally, our companies have been distributing the ThreeLac product for over 10 years, and FiveLac for over 7 years. During this time period, we are not aware of any identified direct link between the use of these products and a significant negative effect on human health, which is supportive testimony to the due diligence of the Founding Scientist in their research and development of the products.

For your reference, our companies have met with international Doctors of Veterinarian Medicine that advocate and use the ThreeLac probiotic blend. In fact and not known to us for the first few years of implementation, ThreeLac has also been utilized in some exotic animals to treat Candidiasis, particularly in dolphin populations for the past 7 or more years. These discussions inspired the development of the capsule product for ThreeLac, which allowed for an easier pathway of inserting the capsule into fish/feed, and then giving the same to the dolphin for ingestion.

Our hope is that you will find this report to be informative in regard to any possible questions that you may have pertaining to the safety of our ThreeLac and FiveLac products. If you have any further questions, please do not hesitate to contact our Customer Service Department at **1-800-305-5710** and ask for our Vice President of Customer Service, Shanna Denfeld.

ght™
The GHT Companies

SNOWDEN CO.,LTD.
3-7-16, Iwamoto-cho, Chiyoda-ku, Tokyo TEL.03-3866-2828

STATEMENT: Safety of Selected Bacteria Strains

July 22, 2014
Snowden Co., Ltd.
Quality Assurance Department

1. This Statement is regarding the following probiotic products manufactured and exclusively distributed (exported) to The GHT Companies by Snowden Co., Ltd. (hereinafter called 'Snowden').

   a. ThreeLac
   b. ThreeLac Capsules
   c. FiveLac

2. This Statement shall focus on the following viable bacteria materials contained in either one or more of the above listed products.

   a. Enterococcus faecalis F-100
   b. Lactobacillus acidophilus
   c. Bacillus coagulans
   d. Bifidobacterium longum
   e. Bacillus subtilis

3. Snowden takes great pride in the development of products that are safe for the consumer; and as a company having such a policy, a precondition of development of the above products was that only viable bacteria listed in the Japanese Pharmaceutical Codex (hereinafter called 'JPC') would be used for the anticipated probiotic formulation and subsequent consumer products. This is based on the fact that all materials listed in JPC are permitted by the Japanese Government due to their safety and effectiveness, and that consumers can rely on JPC when ingesting approved ingredients. JPC is equivalent to USP (U.S. Pharmacopeia) that sets the standards for medicines, food ingredients and dietary supplements in the United States, with said standards being enforced by the FDA (Food

and Drug Administration).

4. Snowden reviewed the viable bacteria listed in JPC and selected the materials to be used, by identifying the viable bacteria (1) used in existing products without reported side effects during the long-term usage, and (2) qualified and approved for use in food products.

5. As the result of comprehensive assessment, the following bacteria strains were identified to meet not only the JPC's criteria, but also the stringent criteria of Snowden: Enterococcus faecalis F-100 and Lactobacillus acidophilus categorized in JPC as Lactomin; Bacillus coagulans categorized in JPC as Spore Forming Lactic Acid Bacteria; Bifidobacterium longum categorized in JPC as Bifidobacterium; and, Bacillus subtilis categorized in JPC as Amylolytic Bacillus.

6. The selected viable bacteria ingredients used in the above listed products (section #1) manufactured and distributed (exported) by Snowden fully conform to JPC standards; and, the all actually used strains are clones of natural bacteria without genetic modification.

7. Snowden selected three approved types of viable bacteria materials for a combination probiotic blend for ThreeLac and ThreeLac Capsules (Enterococcus faecalis F-100; Lactobacillus acidophilus; and Bacillus coagulans), and Snowden selected five approved types of viable bacteria materials for a combination probiotic blend for FiveLac (Enterococcus faecalis F-100; Lactobacillus acidophilus; Bacillus coagulans; Bifidobacterium longum; and Bacillus subtilis). As noted above, a principal reason for the selection of these viable bacteria strains is that they have been used for a long time in Japan, and their effects have been highly evaluated and monitored.

8. Snowden conducted toxicity tests in rats and safety test in vitro to secure and ensure further safety of the selected viable bacteria materials used, and has acquired confirmation that the above viable bacteria materials are fully safe.

9. We have concluded and confirmed that the products listed in section #1 above can be used safely for human consumption and pose no risk to human health.

Masatoshi Sumiya
Deputy General Manager Quality Assurance Department
Snowden Co., Ltd.

Yoshizo Azukabe
President
Snowden Co., Ltd.

SNOWDEN CO.,LTD.
3-7-16, Iwamoto-cho, Chiyoda-ku, Tokyo TEL.03-3866-2828

IN-VITRO SAFETY STUDY on ENTEROCOCCUS FAECALIS F-100

July 22, 2014
Snowden Co., Ltd.
Quality Assurance Department

Introduction

The Enterococci constitute a major genus of the lactic acid bacteria. Some *Enterococci* strains have been used successfully as human probiotics. Their success as probiotics has been attributed to factors such as acid and bile resistance, bile salt hydrolase activity, production of antimicrobials and their ability to survive and compete in the gastrointestinal tract. On the contrary, it has been noted that *Enterococci* have genes coding for antibiotic resistance and virulence determinants; and, some strains of *Enterococci* have also become recognized as pathogens causing bacteremia, endocarditis, and other infections.

Objective

In vitro study was conducted to confirm the safety of the selected strain of *Enterococci*, *Enterococcus faecalis* F-100, used in the probiotics manufactured by Snowden Co., Ltd., such as ThreeLac, ThreeLac capsules, and FiveLac. The study includes the tests to know whether (1) *Enterococcus faecalis* F-100 does not carry problematic virulence determinants, (2) does not have problematic resistance to antibiotics including vancomycin, and (3) does not have hemolytic property. Moreover, by sequencing 16S rRNA, the strain to which *E. faecalis* F-100 should be classified was discussed.

Results

(1) PCR examination of virulence determinants

(Method)

The presence of 14 known potential virulence determinants (*gelE, efaAfm, efaAfs, cpd, cob, ccf, cad, ace, espfm, espfs, aff, cylM cylB, cylA*) in *Enterococci* including *E. faecalis* F-100 were examined with PCR method using 50 ng DNA as template and with respective specific primers[1, 2].

(Result)

The result is summarized in Table 1. Among the 14 possible virulence determinants, only efaAfm and ccf were detected in *Enterococcus faecalis* F-100, whereas multiple virulence determinants were detected in other *E. faecalis*, especially larger number of virulence determinants were detected in two hemolytic *E. faecalis* strains.

Table 1. Detection of virulence determinants in Enterococcus faecalis F-100 and other Enterococci

Virulence determinant	Presence of virulence determinants			
	E. sp. F-100	*E. faecalis ATCC19433*	*E. faecalis DS16* *	*E. faecalis FA22/PAD1* *
gelE	-	+	+	+
efaAfm	+	-	-	-
efaAfs	-	+	+	+
cpd	-	+	+	+
cob	-	+	+	+
ccf	+	+	+	+
cad	-	+	+	+
ace	-	-	+	+
espfm	-	-	-	-
espfs	-	+	-	-
agg	-	-	+	+
cylM	-	-	+	+
cylB	-	-	+	+
cylA	-	-	+	+

*) Hemolytic strains

(Conclusion)
Strain F-100 does not have any potential virulence determinants other than efaAfm and ccf. However, since efaAfm and ccf are present in a large number of *Enterococci* strains of food origin without safety problems [1-4], *E. faecalis* F-100 is thought as safe from virulence determinants point of view.

(2) Resistance to antibiotics

Resistance to various antibiotics including vancomycin of 3 bacterial trains used in ThreeLac (*E. faecalis* F-100, *B. coagulans*, and *B. subtilis*) was examined.

(Method)
Mueller-Hinton agar plates containing 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.031, 0.016, 0.008, 0.004, 0.002, or 0.001 μg/mL were prepared, and 5,000 CFU of each bacterial strain (5 μL), after cultured in Mueller-Hinton broth for 24h at 37ºC, was inoculated onto each plate. Minimum inhibitory concentrations (MICs) were determined after cultivation for 48h at 37ºC.

(Result)
The result is summarized in Table 2. No resistance to any antibiotic used was observed.

Table 2. MICs of various antibiotics against bacterial strains used in ThreeLac

Antibiotics	MIC (μg/mL)		
	E. faecalis F-100	*B. coagulans*	*B. subtilis*
ampicilin	0.5	0.031	0.008
oxacillin	2	0.25	0.031
cefoxitin	2	0.031	0.008
gentamicin	2	0.002	0.25
clarithromycin	4	0.002	0.125
vancomycin	0.25	0.5	0.25
ciprofloxacin	0.5	0.031	0.031
chloramphenicol	8	8	32

(Conclusion)
The results demonstrate that all 3 strains used in ThreeLac are sensitive to 8 different antibiotics examined including vancomycin.

(3) Hemolytic property

It has been suggested that hemolysis of *Enterococci* is associated with their pathogenicity. Therefore, hemolytic properties of the selected *E. faecalis* F-100 was examined.

(Method)
Strain F-100 and other related strains were streaked on Trypticase Soy Agar (BD) plates with 5% horse blood (defibrinated) and cultured for 48 hours at 37˚C to determine the presence of hemolysis.

(Result)
Strain F-100 did now show any hemolytic properties. In contract, other two *E. faecalis* strains tested caused hemolysis.

(Conclusion)
The results demonstrate that strain F-100 is safe to ingest and utilize without hemolytic concerns.

(4) Strain identification of *E. faecalis* F-100 by 16S rRNA-DNA sequencing

Although strain F-100 was originally designated as *Streptococcus faecalis* F-100, afterward this strain was classified as *Enterococci*. To clarify the taxonomic position of strain F-100, DNA encoding 16S rRNA was sequenced.

(Method)
DNA was isolated from strain F-100 and the DNA sequence encoding 16S rRNA was determined with ABI PRISM 3130xl Genetic Analyzer.

(Rusult)
The DNA sequence encoding 16S rRNA of strain F-100 was found to be >99% identity to mostly *E. faecium* strains and also to a few *E. faecalis* strains registered in GenBank.

(Conslusion)
Based on the result of 16S rRNA-DNA sequencing, *E. faecalis* F-100 was the strain very close to *E. faecium*.

Conclusions

In addition to the historical usage of *Enterococcus sp.*, which is included in Japanese Pharmaceutical Codex, the in vitro safety study described in this report shows that this selected strain, *Enterococcus faecalis* F-100, is safe to use in probiotic blends provoking no risk to human health.

References

1. Tuncer, B.O., A.Y. Zeliha, and Y. Tuncer, *Occurence of enterocin genes, virulence factors, and antibiotic resistance in 3 bacteriocin-producer Enterococcus faecium strains isolated from Turkish tulum cheese.* Turk J Biol, 2013. **37**: p. 443-449.
2. Eaton, T.J. and M.J. Gasson, *Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates.* Appl Environ Microbiol, 2001. **67**(4): p. 1628-35.
3. Belgacem, Z.B., et al., *Antimicrobial actiity, safety aspects, and some technological properties of bacteriocinogenic Enterococcus faecium from artisanal Tunisan fermented meat.* Food Control, 2010. **21**: p. 462-470.
4. Noguchi, N., et al., *Characterization of enterococcus strains contained in probiotic products.* Biol Pharm Bull, 2011. **34**(9): p. 1469-73.

Masatoshi Sumiya

Deputy General Manager
Quality Assurance Department
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Yoshizo Asukabe

President
Snowden Co., Ltd.

Brief Explanation for the following documentation:

An Acute Oral Toxicity Study of Enterococcus faecalis

The ten pages of summary reports following this page represent the results of studies jointly performed by Snowden Co., Ltd. and a third party certified testing organization in Japan, by the name of Nippon Experimental Research Institute Co., Ltd.

There were five reviews performed, one on each of the bacteria strains identified in either ThreeLac or FiveLac; specifically, *Enterococcus faecalis, Bifidobacterium longum, Bacillus coagulans, Bacillus subtilis, and Lactobacillus acidophilus*.

With the objective of presenting results that include the actual performance of the testing, we have also included the original summary reports, written in Japanese.

Any questions regarding these results should be directed to **The GHT Companies**, acting exclusively and on behalf of Snowden Co., Ltd.

Report

An Acute Oral Toxicity Study of Enterococcus faecalis in Rats (Limit test)

Study No. H-13086

1. Test substance

(1) Name : Enterococcus faecalis

(2) Lot No. : T1002

2. Guidelines referred

OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. Summary

In clinical signs, no abnormal finding was observed in any animals in test substance group and vehicle group throughout the observation period. And no death was observed in each group.

The body weight increased over the observation period and no significant difference was showed in the mean body weight in the test substance group compared to the vehicle control group.

No abnormal finding was observed in necropsy in any animals in each group.

Based on the above-mentioned results, the approximate LD_{50} value of Enterococcus faecalis was estimated to be more than 2000mg/kg under the conditions of this study.

Nippon Experimental Medical Research Institute Co., Ltd

Study director: Takashi Sato

Date: October 15, 2013

報 告 書

Enterococcus faecalis のラットを用いる急性経口毒性試験(限度試験)

試験番号 H-13086

1. 被験物質
   (1) 名　称　　: Enterococcus faecalis
   (2) Lot No.　　: T1002

2. 参照したガイドライン
   OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. 要　約
   一般状態観察では、観察期間を通して各群全例に異常は認められず、死亡も認められなかった。
   体重では、観察期間を通して増加し、溶媒対照群と比較して当該群の平均体重に有意な差は認められなかった。
   剖検では、各群全例で被験物質に起因すると考えられる異常は認められなかった。
   以上の結果より、本試験条件下における Enterococcus faecalis の概略の LD_{50} 値は、2000 mg/kg を超える用量と推定された。

株式会社 SRD 生物センター
試験責任者　　佐藤　卓

2013 年 10 月 15 日

Report

An Acute Oral Toxicity Study of Bifidobacterium longum in Rats (Limit test)

Study No. H-13086

1. Test substance

(1) Name : Bifidobacterium longum

(2) Lot No. : 2012.09.10

2. Guidelines referred

OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. Summary

In clinical signs, no abnormal finding was observed in any animals in test substance group and vehicle group throughout the observation period. And no death was observed in each group.

The body weight increased over the observation period and no significant difference was showed in the mean body weight in the test substance group compared to the vehicle control group.

No abnormal finding was observed in necropsy in any animals in each group.

Based on the above-mentioned results, the approximate LD_{50} value of Bifidobacterium longum was estimated to be more than 2000mg/kg under the conditions of this study.

Nippon Experimental Medical Research Institute Co., Ltd

Study director: Takashi Sato

Date: October 15, 2013

報 告 書

Bifidobacterium longum のラットを用いる急性経口毒性試験(限度試験)

試験番号 H-13086

1. 被験物質
   (1) 名　称　　: Bifidobacterium longum
   (2) Lot No.　　: 2012.09.10

2. 参照したガイドライン
   OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. 要　約
   一般状態観察では、観察期間を通して各群全例に異常は認められず、死亡も認められなかった。
   体重では、観察期間を通して増加し、溶媒対照群と比較して当該群の平均体重に有意な差は認められなかった。
   剖検では、各群全例で被験物質に起因すると考えられる異常は認められなかった。
   以上の結果より、本試験条件下における Bifidobacterium longum の概略の LD_{50} 値は、2000 mg/kg を超える用量と推定された。

株式会社 SRD 生物センター
試験責任者　　佐藤　卓 ㊞

2013 年 10 月 15 日

Report

An Acute Oral Toxicity Study of Bacillus coagulans in Rats (Limit test)

Study No. H-13086

1. Test substance

(1) Name : Bacillus coagulans

(2) Lot No. : T1002

2. Guidelines referred

OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. Summary

In clinical signs, no abnormal finding was observed in any animals in test substance group and vehicle group throughout the observation period. And no death was observed in each group.

The body weight increased over the observation period and no significant difference was showed in the mean body weight in the test substance group compared to the vehicle control group.

No abnormal finding was observed in necropsy in any animals in each group.

Based on the above-mentioned results, the approximate LD_{50} value of Bacillus coagulans was estimated to be more than 2000mg/kg under the conditions of this study.

Nippon Experimental Medical Research Institute Co., Ltd

Study director: Takashi Sato

Date: October 15, 2013

報 告 書

Bacillus coagulans のラットを用いる急性経口毒性試験(限度試験)

試験番号 H-13086

1. 被験物質
   (1) 名　称　　: Bacillus coagulans
   (2) Lot No.　　: T1002

2. 参照したガイドライン
   OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. 要　約

一般状態観察では、観察期間を通して各群全例に異常は認められず、死亡も認められなかった。

体重では、観察期間を通して増加し、溶媒対照群と比較して当該群の平均体重に有意な差は認められなかった。

剖検では、各群全例で被験物質に起因すると考えられる異常は認められなかった。

以上の結果より、本試験条件下における Bacillus coagulans の概略の LD_{50} 値は、2000 mg/kg を超える用量と推定された。

株式会社 SRD 生物センター

試験責任者　　佐藤　卓 ㊞

2013 年 10 月 15 日

Report

An Acute Oral Toxicity Study of Bacillus subtilis in Rats (Limit test)

Study No. H-13086

1. Test substance

(1) Name : Bacillus subtilis

(2) Lot No. : T1001

2. Guidelines referred

OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. Summary

In clinical signs, no abnormal finding was observed in any animals in test substance group and vehicle group throughout the observation period. And no death was observed in each group.

The body weight increased over the observation period and no significant difference was showed in the mean body weight in the test substance group compared to the vehicle control group.

No abnormal finding was observed in necropsy in any animals in each group.

Based on the above-mentioned results, the approximate LD_{50} value of Bacillus subtilis was estimated to be more than 2000mg/kg under the conditions of this study.

Nippon Experimental Medical Research Institute Co., Ltd

Study director: Takashi Sato

Date: October 15, 2013

報 告 書

Bacillus subtilis のラットを用いる急性経口毒性試験(限度試験)

試験番号 H-13086

1. 被験物質
   (1) 名　称　　: Bacillus subtilis
   (2) Lot No.　　: T1001

2. 参照したガイドライン
   OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. 要　約

一般状態観察では、観察期間を通して各群全例に異常は認められず、死亡も認められなかった。

体重では、観察期間を通して増加し、溶媒対照群と比較して当該群の平均体重に有意な差は認められなかった。

剖検では、各群全例で被験物質に起因すると考えられる異常は認められなかった。

以上の結果より、本試験条件下における Bacillus subtilis の概略の LD_{50} 値は、2000 mg/kg を超える用量と推定された。

株式会社 SRD 生物センター

試験責任者　　佐藤　卓

2013 年 10 月 15 日

Report

An Acute Oral Toxicity Study of Lactobacillus acidophilus in Rats (Limit test)

Study No. H-13086

1. Test substance

(1) Name : Lactobacillus acidophilus

(2) Lot No. : P0801

2. Guidelines referred

OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. Summary

In clinical signs, no abnormal finding was observed in any animals in test substance group and vehicle group throughout the observation period. And no death was observed in each group.

The body weight increased over the observation period and no significant difference was showed in the mean body weight in the test substance group compared to the vehicle control group.

No abnormal finding was observed in necropsy in any animals in each group.

Based on the above-mentioned results, the approximate LD_{50} value of Lactobacillus acidophilus was estimated to be more than 2000mg/kg under the conditions of this study.

Nippon Experimental Medical Research Institute Co., Ltd

Study director: Takashi Sato

Date: October 15, 2013

報 告 書

Lactobacillus acidophilus のラットを用いる急性経口毒性試験(限度試験)

試験番号 H-13086

1. 被験物質
   (1) 名　称　　: Lactobacillus acidophilus
   (2) Lot No.　　: P0801

2. 参照したガイドライン
   OECD Guideline for the Testing of Chemicals, Updating Guideline #420 (Acute Oral Toxicity – Fixed Dose Procedure, Adopted on December 17, 2001)

3. 要　約
   一般状態観察では、観察期間を通して各群全例に異常は認められず、死亡も認められなかった。
   体重では、観察期間を通して増加し、溶媒対照群と比較して当該群の平均体重に有意な差は認められなかった。
   剖検では、各群全例で被験物質に起因すると考えられる異常は認められなかった。
   以上の結果より、本試験条件下における Lactobacillus acidophilus の概略の LD_{50} 値は、2000 mg/kg を超える用量と推定された。

株式会社 SRD 生物センター
試験責任者　　佐藤　卓

2013 年 10 月 15 日

A Study on Inhibition of Biofilm Formation by *Candida albicans*

1. Introduction

Candida sp. is a common fungus species used for making Japanese pickles and fermentation of Japanese Sake. However, some species belonging to *Candida* genus cause serious infectious diseases in some circumstances. In spite of its low pathogenicity in nature, *Candida* shows pathogenicity depending on host factors such as (1) decreased immune-activity in elderly people, (2) prolonged neutropenia due to treatment with anticancer or immunosuppressive agents, and (3) the increased risk of opportunistic infection resulting from long-term use of broad spectrum and multiple antibiotics.

Although the shape of *Candida* cell is spherical, it is said that *Candida* forms biofilms by becoming mycelioid, which makes it more pathogenic.

2. Objective

This study is done to examine if THREELAC is possible to inhibit biofilm formation by *Candida* and consequently prevents *Candida* to become harmful causing candidiasis.

3. Materials

Candida albicans, Lactbacilis sporogenes, Bacillus subtilis, and Enterococcus faecalis F-100 were used as test fungus or bacteria. Sabouraud-dextrose broth DAIGO was used as culture medium to culture in 24 well culture plate. Crystal violet was used to stain *C. albicans*.

4. Method

C. albicans was cultured in Sabouraud-dextrose agar to obtain a single colony. A large single colony was collected with a platinum loop and dispersed in 1 mL of antibiotic-free Sabouraud-dextrose liquid medium. The fluid containing C. albicans was further diluted with liquid medium and mixed with varying concentration of *L. sporogenes, B. subtilis, or E. faecalis* F-100 in each well of 24 well culture plate. After incubated at 30°C for 24 h, the supernatant was removed and the surface of each well was gently washed with purified water. Bodies of *C. albicans* were stained with crystal violet, air-dried, and observed with microscope.

5. Results

Typical photo images of stained *C. albicans* were depicted in Figures 1 to 4. Without addition of bacteria, obvious cellular linkages of *C. albicans* were observed (Figure 1), which was thought to be the initial phase of biofilm formation. On the other hand, if any of the three bacteria included in THREELAC was added, no obvious linkages but only very short ones were observed (Figures 2, 3, and 4), suggesting bacteria contained in THREELAC can break biofilm formation by C. albicans.

6. Conclusion

This study suggests that any of the bacteria contained in THREELAC can inhibit biofilm formation by *C. albicans* and to prevent it to become more pathogenic.

Figures 1 through 4, demonstrating bacterial linkage, continued on next 2 pages.

Figure 1. Bacterial linkages are observed without addition of bacteria

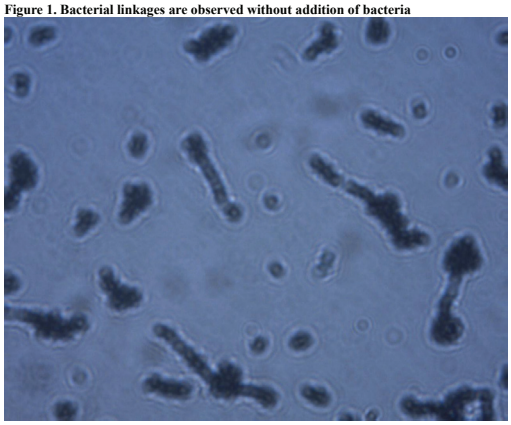


Figure 2. Almost no linkages are observed when *L. sporogenes* was added

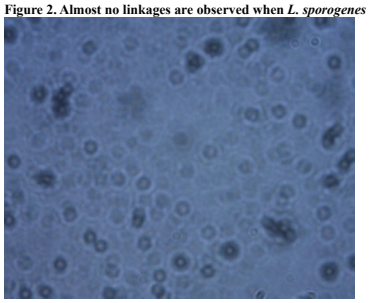


Figure 3. Almost no linkages are observed when *B. subtilis* was added

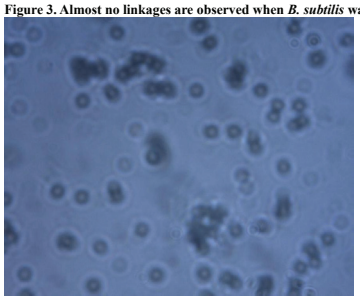


Figure 4. Almost no linkages are observed when *E. faecalis* F-100 was added

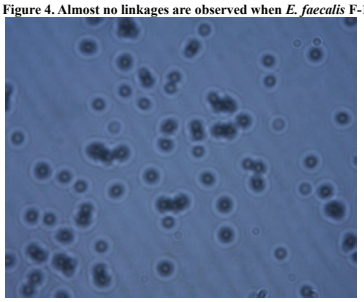


Figure 1. Bacterial linkages are observed without addition of bacteria

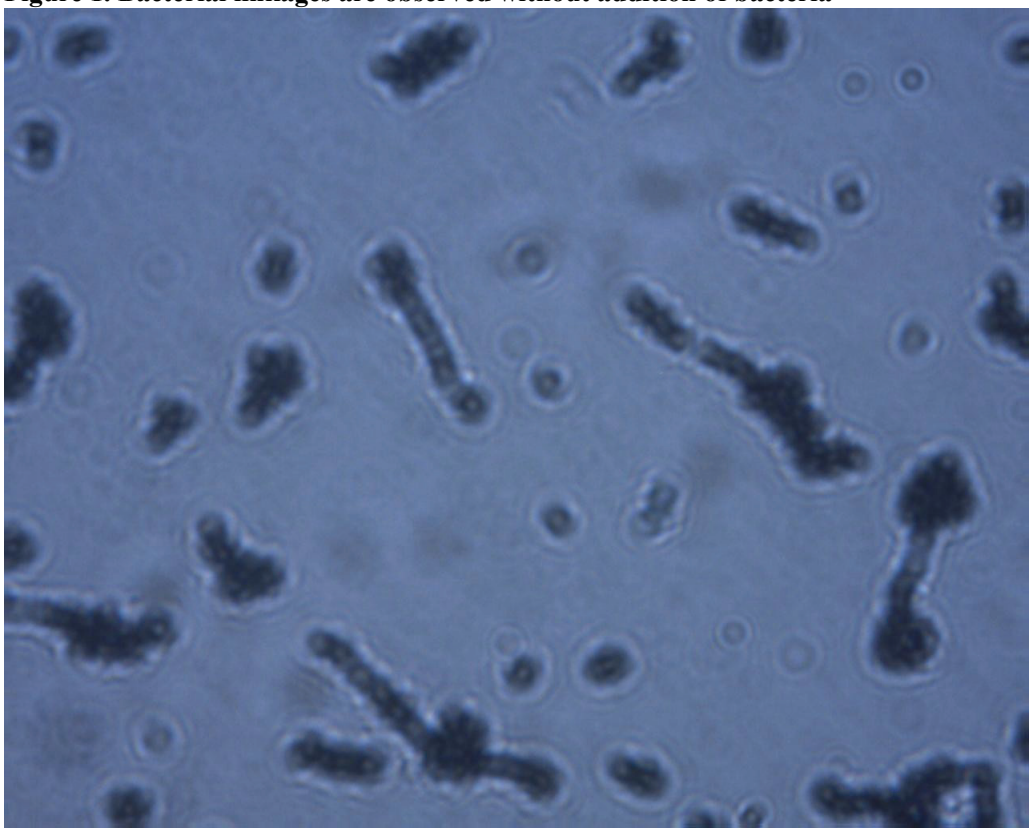


Figure 2. Almost no linkages are observed when *L. sporogenes* was added

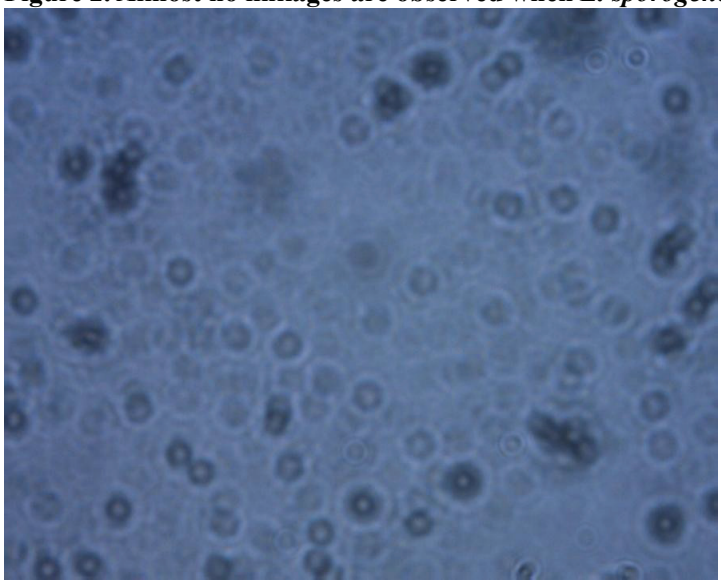


Figure 3. Almost no linkages are observed when *B. subtilis* was added

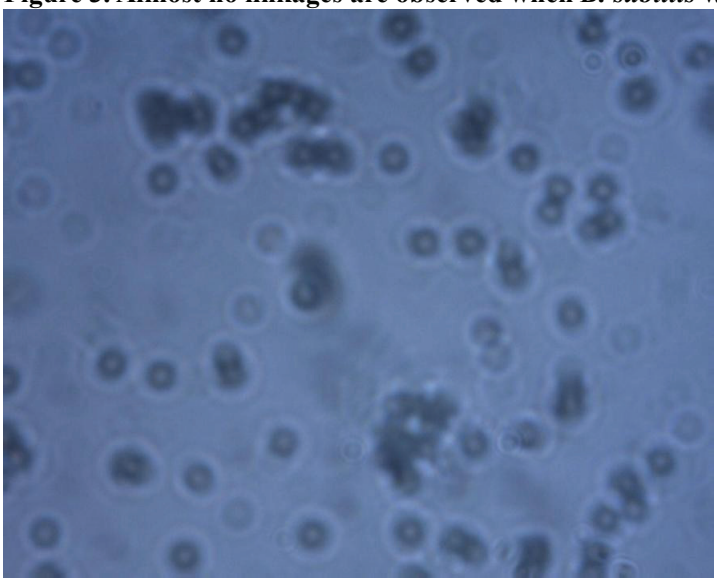
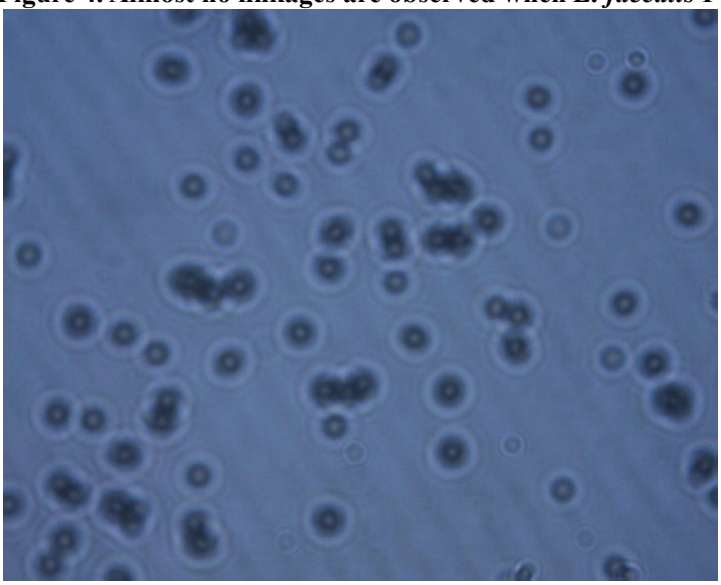


Figure 4. Almost no linkages are observed when *E. faecalis* F-100 was added



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The signatures below represent review and confirmation of A Study on Inhibition of Biofilm Formation by Candida albicans and acknowledgement of the work effort, stated results and applicable conclusions.

Sumiya Masatoshi

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Clinical Survey on the Effects of THREELAC in humans, *including the impact on selected systemic conditions identified in the small and large intestines, as identified in testing of fecal matter.*

1. Introduction

The principal active ingredients in THREELAC, a probiotic blend product, are *Bacillus coagulans, Enterococcus faecalis* (strain F-100) and *Bacillus subtilis*. THREELAC further contains complimentary ingredients such as refined dry beer yeast powder and dietary fiber (galactomannan). It is well known that "probiotics" contribute to the improvement of the balance of the intestinal microbial flora, specifically *Candida albicans* species, and the enhancement of short-chain fatty acid (SCFA) production in human gut.

2. Purpose

This initial small clinical study was planned and conducted using a randomized, placebo-controlled approach in order to confirm positive health contributions resulting from the ingestion of THREELAC.

3. Method (Test protocol)

1) The test group taking THREELAC (Test Group) and the control group taking placebo (Control Group) are selected. Each group has 12 members of which gender and age are random.
2) Feces of all the participants are collected before starting test.
3) Test Group takes THREELAC immediately following each meal, three times per day, during the test period of four weeks. Control Group takes placebo in the same manner and during the same period. Placebo is prepared eliminating the three major active ingredients described above in THREELAC.
4) All the participants record and report the color, appearance, and odor of feces during the test period. Frequency of the bowel movements per day during the period is also reported.
5) Feces of all the participants are collected again just after the test period.
6) The species type and numbers of bacteria found and identified in the collected feces are analyzed by "Agar Plate Assay" in accordance with Mitsuoka's method [1].
7) SCFA and putrefactive production in the collected feces are measured by gas-chromatography.

4. Results

1) No significant differences in the color, appearance, and odor of feces were observed between Test and Control Groups (Figure 1).2) Three participants in Test Group reported that the frequency of bowel action was improved and regular bowel action almost every day was observed. (Table 1)

2) Three participants in Test Group reported that the frequency of bowel action was improved and regular bowel action almost every day was observed. (Table 1)
3) The statistically significant difference in the increase of *Bifidobacteria* in the feces collected after the test was observed between Test and Control Groups. (Figure IC)
4) The statistically significant difference in the decrease of *Candida sp*. and *Clostridium perfringens* (generally called Welsh bacterium) in the feces collected after the test was observed between Test and Control Groups. (Table 2)
5) No significant difference in SCFA production was observed between before and after ingestion of THREELAC. (Figure 2)
6) No safety problems were observed by ingesting THREELAC.

5. Conclusions

Referring to the test results described above, we estimate that THREELAC contributes to the improvement of intestinal microbial flora and consequently regular bowel action without safety problems.

Reference

1. Hida M, Aiba Y, Sawamura S, Suzuki N, Satoh T, Koga Y. Inhibition of the accumulation of uremic toxins in the blood .and their precursors in the feces after oral administration of Lebenin, a lactic acid bacteria preparation, to uremic patients undergoing hemodialysis. Nephron. **74**, 349-55. (1996)

Tables 1 and 2, and Figures 1 and 2, are continued on next 5 pages.

Improved Items	Control Group	Test Group
lack or Dark to Light Brown)	1 / 12	0 / 12
ınce (Fluid to Solid)	2 / 12	2 / 12
ronger to Softer)	3 / 12	2 / 12
ction (Ever 2 days or more to everyday)	0 / 12	3 / 12

Table 1. Improvement of the conditions of feces

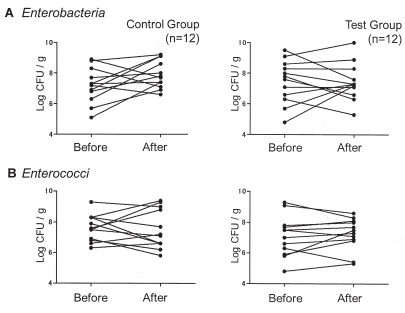

Figure 1. Improvement of Microbial Flora by THRELAC

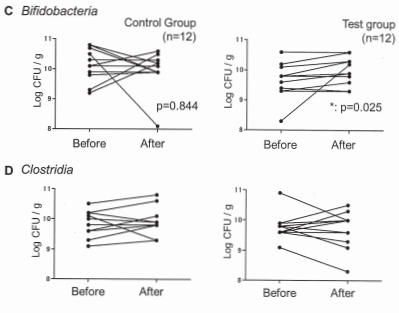

Figure 1. Improvement of Microbial Flora by THRELAC (Continued)

	Control Group	Test Group	Fisher's exact test (one- tailed)
Pseudomonas sp.	6 / 12 → 5 / 12	3 / 12 → 2 / 12	
Staphylococcus sp.	4 / 12 → 1 / 12	3 / 12 → 3 / 12	
Candida sp.	5 / 12 → 4 / 12	8 / 12 → 3 / 12	*: p=0.050
Clostridium perfringens	5 / 12 → 3 / 12	6 / 12 → 1 / 12	*: p=0.035

Table 2. Reduction of injurious microorganism by THREELAC

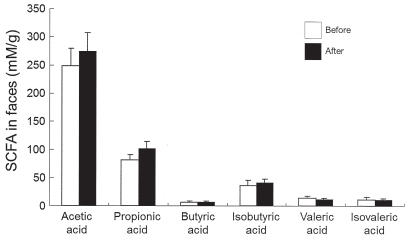

Figure 2. Effect on SCFA Production of THREELAC

Improved Items	Control Group	Test Group
Color (Black or Dark to Light Brown)	1 / 12	0 / 12
Appearance (Fluid to Solid)	2 / 12	2 / 12
Odor (Stronger to Softer)	3 / 12	2 / 12
Bowel Action (Ever 2 days or more to everyday)	0 / 12	3 / 12

Table 1. Improvement of the conditions of feces

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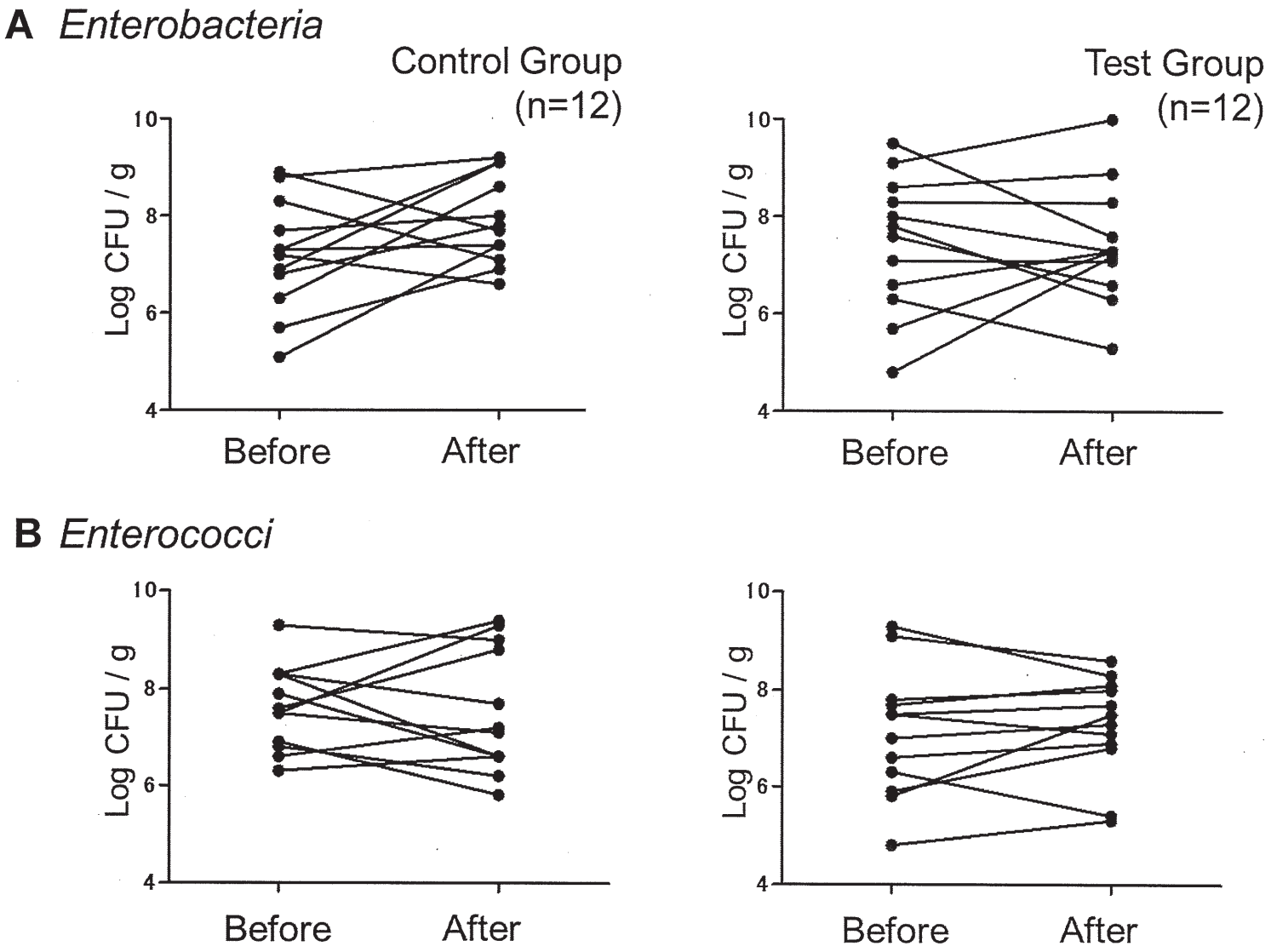

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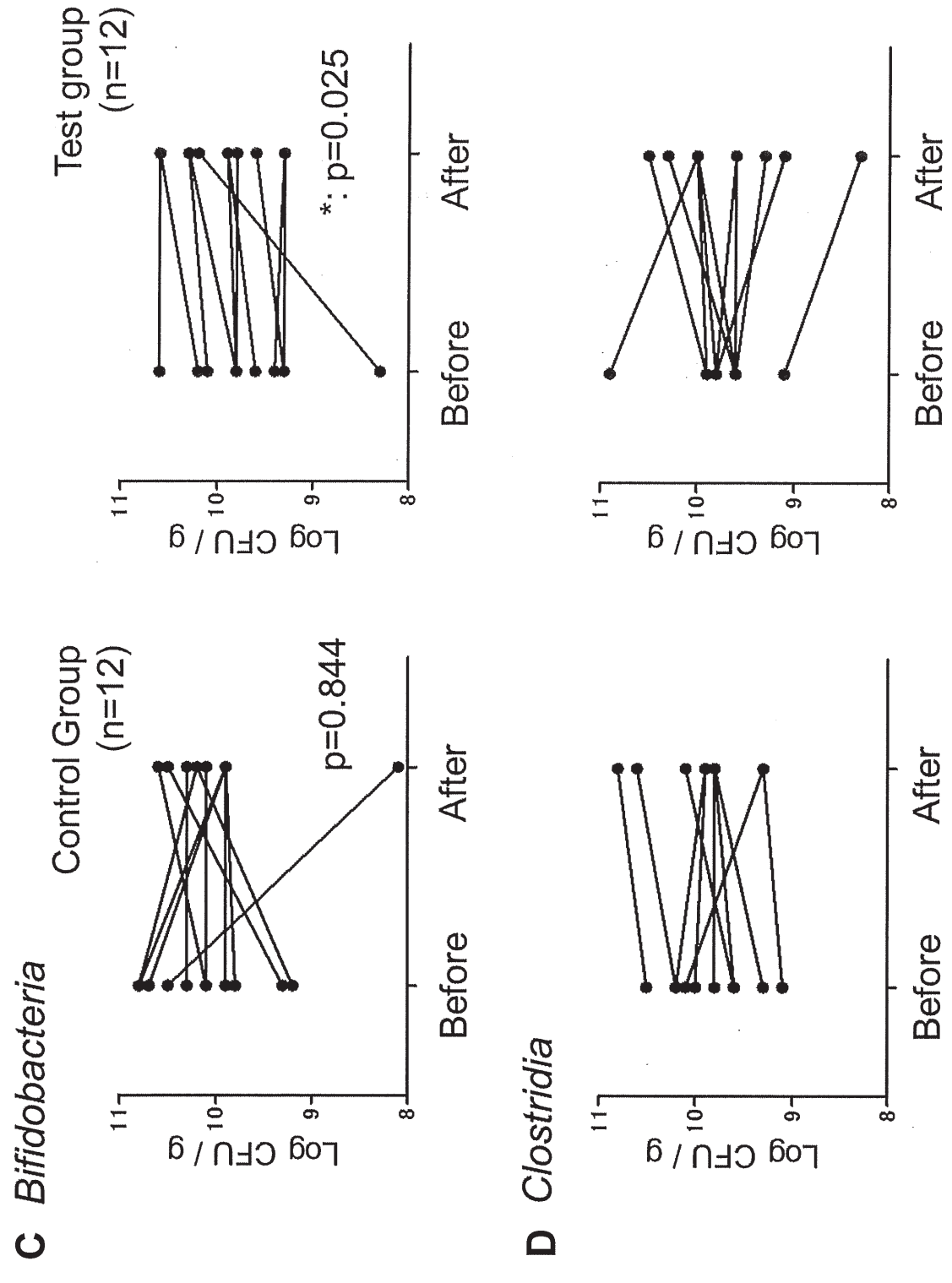

Figure 1. Improvement of Microbial Flora by THRELAC (Continued)

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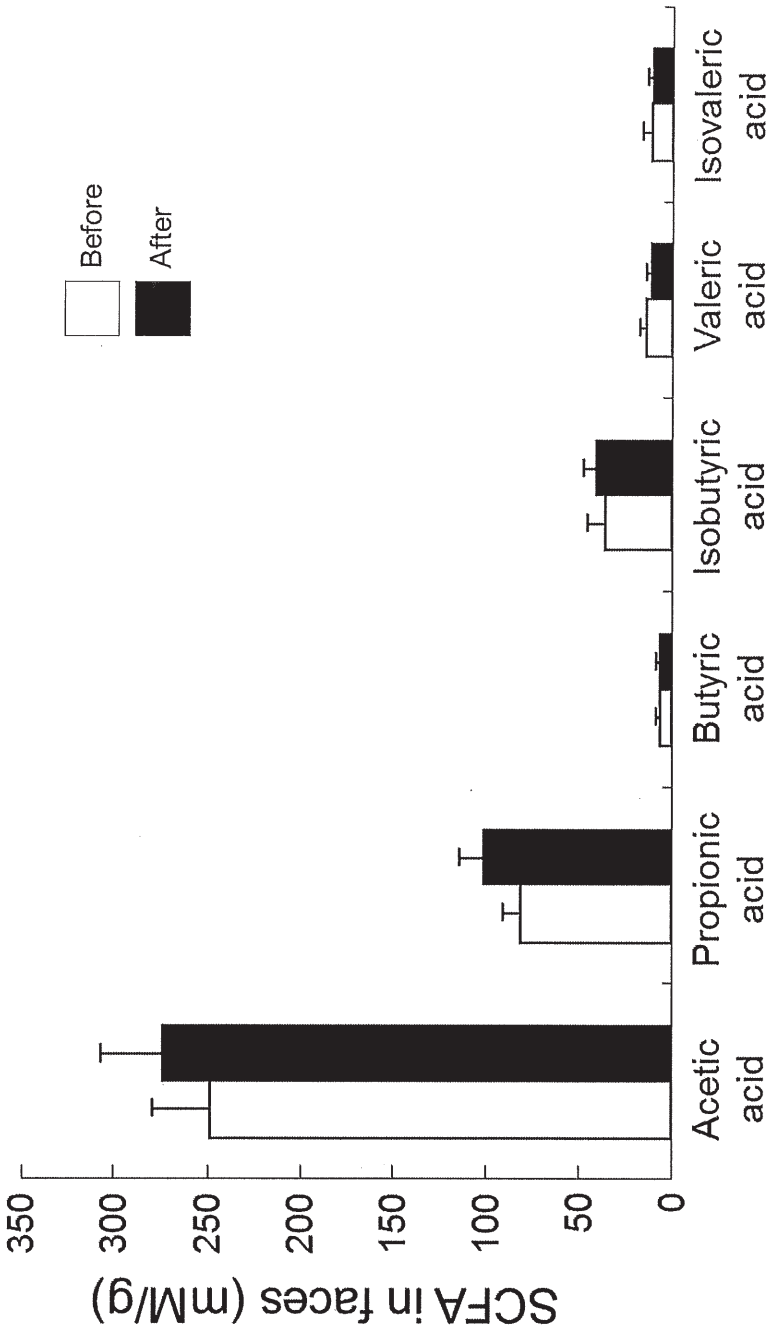

Figure 2. Effect on SCFA Production of THREELAC

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The signatures below represent review and confirmation of Clinical Survey on the Effects of THREELAC in humans and acknowledgement of the work effort, stated results and applicable conclusions.

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