American Institute of Integrative Oncology
RESEARCH & EDUCATION

Foundations of Integrative Oncology
COURSE

**GRAND ROUNDS CALL**
**With Dr. Nalini Chilkov**
**September 18th, 2019**
Second Wednesday of Every Month
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

**Agenda**

- Clinical Pearl:
  - Managing Menopausal Symptoms in Cancer Patients with Chinese Medicine

- Clinical Questions:
  - Regarding lymphedema

- Research Highlights:
  - PTSD linked to increased risk of ovarian cancer
  - Strategies to promote abundance of Akkermansia muciniphila, an emerging probiotics in the gut, evidence from dietary intervention studies
  - Manipulating Gut Microbiota Composition to Enhance the Therapeutic Effect of Cancer Immunotherapy
  - Effect of Selected Stilbenoids on Human Fecal Microbiota
  - Metabolism of Stilbenoids by Human Faecal Microbiota
  - The gut microbiome and response to immune checkpoint inhibitors: preclinical and clinical strategies

| **Clinical Pearl: Managing Menopausal Symptoms in Cancer Patients with Chinese Medicine** |
|---|
| **See Resource Library for Slides and Recording** |

| **Case Study:** |
|---|
| No Case Study Submitted |

| **Questions & Answers** |
|---|
| Susie Thomson - **Regarding lymphedema, I have a client who is suffering in her lower leg after a full hysterectomy with several lymph nodes removed, she has support stockings, she raises it and she is having lymphatic drainage massages twice per week. I have put her on a anti-inflammatory diet, are there any other suggestions which may help? Any supplements which might help? In fact, the** |

**physiotherapist was reluctant to do the drainage massage. She was worried this might spread cancer cells, a common belief here, I said it was unlikely, I hope I am right? So, two questions I guess! Any advice would be greatly appreciated"**

**Dr. Chilkov Response:**
Lymphedema of the lower extremities is very difficult to manage because gravity is always moving the fluids downwards. Mechanical measures such as elevating the legs above the level of the heart if possible (when lying) and getting up to move every hour. While sitting or lying in bed flex the feet frequently. If at a desk get an under the desk bicycle exerciser to keep the legs moving Wear appropriate compression stockings, gentle lymphatic massage is helpful (No deep tissue massage)

When submitting a question please submit a complete history is presented (by completing our case history form),
In the OUTSMART CANCER SYSTEM we practice highly individualized care
There is never a simply formulaic answer to any question. We treat the whole biosystem, the whole person, not the presenting signs and symptoms. (in this case lymphedema of the lower extremity)

In the future please complete a case history form with supporting medical records for a thorough assessment and appropriate interventions

In order to evaluate the whole person, the whole biosystem, contributing factors and etiology much more information must be provided

What is her age
What is her lifestyle Is she able to exercise?
What is her diet
What is her emotional and psychological state?

Does she have hypertension
Is she obese
Does she have a stress or sleep disorder
Does she have cardiovascular or kidney dysfunction
Does she have hypothyroidism
Is she on medications that cause retention of fluids and edema
Does she have varicose veins
Does she have adhesions in her pelvis from prior treatments or surgeries
Does she have ascites
Does she have peritonitis
ETC ETC

A very generalized answer that is not individualized is all that can be offered without a detailed history.

A thorough clinician practicing highly individualized care will consider all factors listed below and determine how the whole biosystem can be addressed. This is the art of medicine. To find the pattern that leads to the presenting signs and symptoms

If she does not have kidney dysfunction or kidney disease she can remove all added salt from her diet, consume 40-50 grams of protein daily. Eat a ketogenic diet strictly 2 days each week, eat the low glycemic OUTSMART CANCER Diet on the other 5 days and practice intermittent fasting for 13-16 hours per 24 hours every night.

Take measures to reduce edema and produce diuresis
Includes mechanical and dietary guidelines above

Address Inflammation:
Omega 3 Fatty acids 2-4g/day, with food
DFH Inflammatone 3 caps 4x/day on empty stomach (Curcumin, Boswellia, proteases)

Support health of blood vessels and connective tissue
Foods high in phytophenols daily
Quercetin + Bromelain supplement 500mg 4x/day

Support normal bowel function
Plant based high fiber diet
Support healthy microbiome

Normal diuresis can be enhanced safely with above interventions and
Non irritating potassium sparing botanicals such as Dandelion Root (Rdx Taraxacum) , Burdock (Rdx Arcticum) Root, Parsley Root (Rdx Petroselinum), Black Cumin Seed Oil (Nigella sativa)l

Pyridoxal 5 Phosphate 50 mg once daily (monitor serum VIT B6 levels. . Do not overdose)

Traditional Chinese Herbal Diuretic Formula Wu Ling San (listed on cancer.gov/publications)
Protective to Kidney Function .
induces diuresis and natriuresis via inhibition of the renin-angiotensin-aldosterone system

A traditional Chinese medicine (TCM) composed of Polyporus sclerotium (Sclerotium Polypori Umbrellati; Zhu Ling), hoelen (Poria; Sclerotium Poriae Cocos; Fu Ling), Alismatis rhizome (Alisma; Rhizoma Alismatis Orientalis; Ze Xie), Cinnamomi cortex (Ramulus Cinnamomi Cassiae; Gui Zhi) and Atractylodis macrocephalae rhizome (Rhizoma Atractylodis Macrocephalae; Bai Zhu) with potential diuresis-inducing and kidney-protective activities. Upon oral administration, wu-ling san may increase the removal of excess fluid, prevent the retention of water, maintain healthy water metabolism by promoting diuresis, and protect kidney function.

**Recommendations from**
**Dr. Warren Ross MD**: Exercise in water up to chest for benefit of hydrostatic pressure

**Judy Pruzynski L.Ac**.: Medical Chi Gong with an experienced practitioner who know how to open and close joints She recommends Chi Kung practitioners who have been trained Bruce Frantzis. He lists qualified practitioners on his website energyarts.com

**There may be a prior Clinical Pearl on Lymphedema**
**Check the resource library**

## Research: PTSD linked to increased risk of ovarian cancer

MIND BODY CONNECTION - know your patient
**Women who experienced six or more symptoms of post-traumatic stress disorder (PTSD) at some point in life had a twofold greater risk of developing ovarian cancer compared with women who**

**never had any PTSD** symptoms, according to a new study from researchers at Harvard T.H. Chan School of Public Health and Moffitt Cancer Center.

The findings indicate that having higher levels of PTSD symptoms, such as being easily startled by ordinary noises or avoiding reminders of the traumatic experience, can be associated with increased risks of ovarian cancer even decades after women experience a traumatic event. The study also found that t**he link between PTSD and ovarian cancer remained for the most aggressive forms of ovarian cancer.**

**The findings were published in Cancer Research, on September 5, 2019.**

Andrea L. Roberts, Tianyi Huang, Karestan C. Koenen, Yongjoo Kim, Laura D. Kubzansky, Shelley S. Tworoger.
Posttraumatic stress disorder (PTSD) is associated with an increased risk of ovarian cancer: a prospective and retrospective longitudinal cohort study.
Cancer Research, 2019; canres.1222.2019 DOI: 10.1158/0008-5472.CAN-19-1222

**ABSTRACT**

Ovarian cancer is the deadliest gynecologic cancer. **Chronic stress accelerates tumor growth** in animal models of ovarian cancer. We therefore postulated that posttraumatic stress disorder (PTSD) may be associated with increased risk of ovarian cancer. We used data from the Nurses' Health Study II, a longitudinal cohort study with **26 years of follow up, conducted from 1989-2015 with 54,710 subjects.** Lifetime PTSD symptoms were measured in 2008. Self-reported ovarian cancer was validated with medical records. Risk of ovarian cancer was estimated with Cox proportional hazards models and further adjusted for known ovarian cancer risk factors (e.g., hormonal factors) and health risk factors (e.g., smoking). Fully prospective secondary analyses examined incident ovarian cancer occurring after PTSD assessment in 2008. Additionally, we examined associations by menopausal status. During follow-up, 110 ovarian cancers were identified. **Women with high PTSD symptoms had 2-fold greater risk of ovarian cancer versus women with no trauma exposure** (age-adjusted hazard ratio (HR)=2.10, 95% confidence interval (CI)=1.12, 3.95). Adjustment for health- and ovarian-cancer risk factors moderately attenuated this association (HR=1.86, 95% CI=0.98, 3.51). Associations were similar or moderately stronger in fully prospective analyses (age-adjusted HR=2.38, 95% CI=0.98, 5.76, N cases=50) and in premenopausal women (HR=3.42, 95% CI=1.08, 10.85). In conclusion, we show that **PTSD symptoms are associated with increased risk of ovarian cancer**. Better understanding of the underlying molecular mechanisms could lead to interventions that reduce ovarian cancer risk in women with PTSD and other stress-related mental disorders.

**Research: Strategies to promote abundance of Akkermansia muciniphila, an emerging probiotics in the gut, evidence from dietary intervention studies**

Strategies to promote abundance of Akkermansia muciniphila, an emerging probiotics in the gut, evidence from dietary intervention studies
J Funct Foods. 2017 June ; 33: 194–201. doi:10.1016/j.jff.2017.03.045.
Kequan ZhouDept of Nutrition & Food Science, Wayne State Univ, Detroit, MI 48202, USA

**ABSTRACT**

**Akkermansia muciniphila is a mucin-degrading bacterium commonly found in human gut.** A. muciniphila has been **inversely associated with obesity, diabetes, inflammation, and metabolic disorders.** Due to its highly promising probiotic activities against obesity and diabetes, A. muciniphila has drawn intensive interest for research and development in recent years. A number of human and animal studies have shown that **the abundance of A. muciniphila in the gut can be enhanced through dietary interventions.** The present review focuses on evidence-based dietary strategies of improving A. muciniphila abundance in the gut by critically appraising up-to-date available human and animal intervention studies on A. muciniphila growth and their impact on risk factors of obesity and diabetes. Their potential mechanisms in promoting A. muciniphila are also discussed along with the discussions of mechanism of action for A. muciniphila to exert probiotic functions.

**PROMOTING AM in the Intestines**

- **Supplement with AM**

- **Supplement with Selected Probiotics**
  - Bifidobacterium animalis lactis
  - Lactobacillus rhamnosus
  - Increase Ratio of Firmicutes to Bacteroidetes

- **Supplement with PreBiotic Fructooligosaccharides (FOS)**
  - **Fructooligosaccharides** (FOS) are **oligosaccharides** that occur naturally in plants such as onion, chicory, garlic, asparagus, bananas, artichoke, among many others. They are composed of linear chains of **fructose** units, linked by beta (2-1) bonds.

- **Implement Low FODMAP Diet** (Fermentable Oligo-, Di- and Mono-saccharides and Polyols)
  - Includes fructose, lactose, oligosaccharides, polyols, sugar alcohols
  - **Low FODMAP diet** was associated with higher fecal pH, greater microbial diversity, normal SCFA and reduced adverse gastrointestinal symptoms
  - **Polyphenols** (mixed studies) used cranberry and concord grape polyphenols
    - Resveratrol and stilbenes impact microbiome
  - **Metformin** (AM is decreased in diabetic and insulin resistant obese patients)
  - **Chinese Rhubarb Root, Rheum palmatum (Da Huang)** anthraquinones thought to be active principle
    - Also hepatoprotective
  - **Caloric Restriction-Intermittent Fasting**
  - Selective antibiotics (AM resistant to Vancomycin): Not applicable to our approach due to disruption of whole biosystem
  - **Moderate** Exercise
  - **Include Caffeic Rich Foods** in Diet (see list below)

- **Factors that Reduce Abundance of AM**
  - High fat diet (Ketogenic and Paleo diets tend to be low in plant fibers)
  - Alcohol Consumption
  - Insulin Resistance
  - Obesity

- Increased inflammatory biomarkers (TNFa, IL6, NFkB)

**FOODS RICH IN CAFFEIC ACID (not related to caffeine)**

Increases AM
Increases Firmicutes to Bacteriodes ratio
Reduces inflammation biomarkers (NFkB, IL6, TNFa)

- Coffee
- wine
- turmeric
- basil
- thyme
- oregano
- sage
- cabbage
- apples
- strawberries
- cauliflower
- radishes
- mushrooms
- kale
- pears
- olive oil

## Research: Manipulating Gut Microbiota Composition to Enhance the Therapeutic Effect of Cancer Immunotherapy

Ming Yi, MD1 , Dechao Jiao, MD, PhD2 , Shuang Qin, MD1 , Qian Chu, MD, PhD1 , Anping Li, MD2 , and Kongming Wu, MD, PhD
Integrative Cancer Therapies Volume 18: 1–13 DOI: 10.1177/1534735419876351

**Abstract**

In the past decade, a growing set of immunotherapies including immune checkpoint blockade, chimeric antigen receptor T cells, and bispecific antibodies propelled the advancement of oncology therapeutics. Accumulating evidence demonstrates that immunotherapy could eliminate tumors better than traditional chemotherapy or radiotherapy with lower risk of adverse events in numerous cancer types. Unfortunately, a substantial proportion of patients eventually acquire resistance to immunotherapy. **By analyzing the differences between immunotherapy-sensitive and immunotherapy-resistant populations, it was noticed that the composition of gut microbiota is closely related to treatment effect.** Moreover, in

xenograft models, interventional regulation of gut microbiota could effectively enhance efficacy and relieve resistance during immunotherapy. Thus, **we believe that gut microbiota composition might be helpful to explain the heterogeneity of treatment effect, and manipulating gut microbiota could be a promising adjuvant treatment for cancer immunotherapy.** In this mini review, we focus on the latest understanding of the **cross-talk between gut microbiota and host immunity**. Moreover, we highlight **the role of gut microbiota in cancer immunotherapy** including immune checkpoint inhibitor and adoptive cell transfer.

- **Bifidobacterium** could enhance the function of dendritic cells-DC by promoting DC maturation, upregulating cytokine secretion, stimulating DC-IL-12-Th1-skewing immune response, as well as facilitating the activation and survival of tumor specific T cells.

- ...**Patients responding to nivolumab therapy possessed higher diversity of gut microbiota at baseline, which sustained stable composition during treatmen**t.108 Composition difference analysis between responder group and nonresponder group showed that bacteria such as Alistipes putredinis, B longum, and Prevotella copri were significantly enriched in responders, while unclassified Ruminococcus were enriched in nonresponders….

- ….antibiotics could reshape the composition of gut microbiota that further interferes with the effect of immunotherapy, t**he relationship between antibiotic-associated dysbiosis and immunotherapy is another hot topic….**

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# HIGHLIGHTS:
# Managing Menopausal Symptoms in Cancer Patients with CHINESE MEDICINE

Dr. Nalini Chilkov, Founder

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## Menopause Accelerated Due to Cancer Treatment

- Chemotherapy Induced Ovarian Failure
- Radiation Therapy to Pelvis
- Hormone Therapy
- Ovarian Suppression Leuprolide (Lupron) or goserelin (Zoladex)
- Aromatase Inhibitors Letrozole, Anastrozole, Exemestane)
- Tamoxifen (Selective Estrogen Receptor Modulator)\
- Fulvestrant. (Degradation of Estrogen Receptor)
- Ovarian Surgery Oophorectomy

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## Common Menopausal Symptoms

- Vasomotor Symptoms (Hot Flashes)
- Sleep Disturbances
- Urinary Incontinence
- Vulvovaginal Atrophy
- Loss of Libido
- Depression
- Joint Pain
- Osteopenia-Osteoporosis

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# Traditional Chinese Herbal Formulas for Menopausal Syndrome

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## Er Xian Decoction

**a famous Chinese herbal prescription,**
unique effect on osteoporosis and menopausal syndrome

- **Rhizoma Curculiginis**
- **Herba Epimedii**
- **Radix Morindae Officinalis**
- **Radix Angelicae Sinensis**
- **Cortex Phellodendri**
- **Rhizoma Anemarrhenae**

DOSE
3 grams freeze dried granules
Twice daily
Or
3 capsules

For decades, Erxian Decoction has been widely used to improve various menopausal symptoms, such as hot flushes, night sweats, insomnia, and depression, due to its definite therapeutic effect with no severe adverse reactions reported

J Ethnopharmacol. 2019 Apr 24;234:8-20. doi: 10.1016/j.jep.2019.01.010.
**Erxian decoction, a Chinese herbal formula, for menopausal syndrome: An updated systematic review.** Wang YL, Lou XT, Shi YH, Tong Q, Zheng GQ.

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## XIAO YAO San (Bupleurum & Tang Kuei Formula)
## Free and Easy Wanderer Formula

3 grams twice daily

| | |
|---|---|
| Chai Hu | Rdx Bupleurum falcatum |
| Dong Quai. | Rz Angelica sinensis |
| Bai Shao | Rdx Paeonia alba |
| Bai Zhu | Rdx Atractylodes alba |
| Fu Ling Pi | Sclerotum Poria Cocos |
| Bo He | H Mentha piperita |
| Gan Jiang | Rz Zingiberis |
| Gan Cao | Rdx Glycyrrhiza glabra |

MODIFIED
Jia Wei Xiao Yao San
Moutan & Gardenia Rambling Powder
(addresses more heat symptoms and more agitation)

Add
Mu Dan Pi Cortex Paeonia suffruticosa
Zhi Zi Fr. Gardenia

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## Modified Xiaoyao Powder's therapeutic effects on breast cancer patients receiving tamoxifen

A 10-year analysis of 20,466 breast cancer patients treated with tamoxifen showed that more than half the subjects had ever used CMs, in which modified Xiaoyao Powder had the highest utilization rate, nearly one-third. The analysis showed that application of CMs reduced the risk of endometrial cancer induced by tamoxifen (Tsai et al., 2014).

A randomized controlled trial (Sun and Zhang, 2013) observed **Modified Xiaoyao Powder's therapeutic effects on breast cancer patients receiving tamoxifen** and enrolled 31 patients administered with the modified Xiaoyao Powder and 30 cases with tamoxifen alone. After 2 months of treatment, the Kupperman Index of the modified Xiaoyao Powder group was
significantly lower than that of the control group.

**Modified Xiaoyao Powder did not affect the estrogen levels.**

Other clinical studies (Xu et al., 2005; Zhang and Zheng, 2012; Fu, 2016; (Zhao et al., 2017) also showed that **Modified Xiaoyao Powder produced some improvement of perimenopausal symptoms of breast cancer and had good safety;**



## Heavenly Emperor Formula
## Tian Wang Bu Xin Tang

Yin Nourishing: Disharmony of Heart and Kidney
Enriches the yin, nourishes the blood, tonifies the Heart, and calms the spirit

DOSE
3 grams freeze dried granules Twice daily
Or
3 capsules twice daily

**Rehmannia Root** (Shu di huang) 6.86
Ginseng (Ren shen) 0.86
Chinese Senega Root (Yuan zhi) 0.86
Radix Scrophulariae (Xuan shen) 0.86g
**Biota orientalis seed** (Bai zi ren) 3.43g
Platycodon Root (Jie geng) 0.86g

**Asparagus Tuber** (Tian men dong) 3.43g
Salvia miltiorrhiza (Dan shen) 0.86g
**Zizyphus spinosa** (Suan zao ren) 3.43g
**Tuber ophiopogonis japonici** (MaiMen Dong) 3.43g
Hoelen (Fu ling) 0.86g
**Angelica sinensis** (Dang gui) 3.43g
Schisandra fruit (Wu wei zi) 0.86g



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Shuo Wang et al

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**Chinese herbal medicine for menopausal symptoms**
Xiaoshu Zhu et al

## Dr. Nalini's Menopausal Acupuncture Points

| | | |
|---|---|---|
| Liv 3 | St 36 | GV 4 |
| K3 | Sp 6 | GV 20 |
| H7 | | |
| PC6 | Kid 6 | ESM |
| | Lu 7 | |
| | Kid 16 | |
| | K27 | |



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## MENOPAUSAL VASOMOTOR SYMPTOMS HOT FLASHES

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## MENOPAUSAL SLEEP DISRUPTION



## Three Component Herbal Tea
## Alleviates Prolonged Fatigue and Improves Sleep Quality
## A Randomized Controlled Study

| | | |
|---|---|---|
| Astragalus Radix. | Huang Qi | 1200mg |
| Angelica gig.Radix | Dang Gui. | 900mg |
| Zizypus Fructus. | Da Zao. | 900mg |

**3 grams freeze dried herbal tea pellets twice daily**
**At 8 weeks;**
**Significant improvement in sleep quality, fatigue and depression**

EXPLORE November/December 2018, Vol. 14, No. 6

## Dr. Nalini's Menopausal Insomnia Points

Liv 3
H7 PC6
K6 Lu 7  Kid 16
Sp 6
ESM



## Selected References: Acupuncture, Menopause, Insomnia

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## AROMATASE INIHIBITORS and JOINT PAIN

## Dr. Nalini's Acupuncture Points and Liver Yin Nourishing Herbs for Aromatase Inhibitor Related Joint Pain

St 36, Liv 3, GB 34, Sp 6, Sp 3 , Local Points

Liver Yin Tendon Nourishing Herbs
Rz. Rehmannia glutinosa
Paeonia alba, Fr. Schizandra, Fr. Lycium



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## MENOPAUSE and DEPRESSION

## Adjuvant Therapy of Oral Chinese Herbal Medicine for Menopausal Depression: A Systematic Review and Meta-Analysis

**Bupleuri Radix (Chinese name: Chaihu) and Paeoniae Radix Alba (Chinese name: Baishao) were the herbs with top frequency.**

**Bupleurum-saikoside, the main active ingredient of Bupleuri Radix, improved depression by regulating the monoamine neurotransmitters and BDNF in the brain**

**Paeoniae Radix Alba improved depression by increasing the single amine neurotransmitter and adjusting the dysfunction of HPA axis**

Evidence-Based Complementary and Alternative Medicine
Volume 2018, Article ID 7420394, 14 pages. Jiju Wang, et al. Murine study



J Psychiatr Res. 2014 Oct;57:165-75

## A systematic review on the efficacy, safety and types of Chinese herbal medicine for depression.

Yeung WF[1], Chung KF[2], Ng KY[3], Yu YM[4], Ziea ET[5], Ng BF[6]

DOSE
3 grams freeze dried granules twice daily

The frequently used formulas were
- Xiao Yao- Bupleurum and Peony decoction
- Chaihu Shugan- Bupleurum and Cyperus decoction
- Ganmai Dazao Licorice, Wheat berry and Jujube decoction

Chaihu (Bupleurum) , Bai Shao (Paeonia alba) and Fu Ling (Poria cocos) were the frequently used single herbs

Meta-analyses showed that CHM monotherapy was better than placebo and as effective as antidepressants in reducing Hamilton Depression Rating Scale (HDRS) score



## Dr. Nalini's ACUPUNCTURE POINTS Menopausal Depression

| | | |
|---|---|---|
| Liv 3 | St 36 | GV 4 |
| K3 | Sp 6 | GV 20 |
| H7 | | |
| PC6 | Kid 6 | ESM |
| | Lu 7 | |
| | Kid 16 | |

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Biomed Res Int. 2018 May 29;2018:5351210. doi: 10.1155/2018/5351210. eCollection 2018.



## MENOPAUSAL VULVOVAGINAL ATROPHY



## Comparison between vaginal royal jelly and vaginal estrogen effects on quality of life and vaginal atrophy in postmenopausal women: a clinical trial study

Electronic Physician http://www.ephysician.ir
November 2016, Volume: 8, Issue: 11, Pages: 3184-3192,
Fatemeh Seyyedi, et al

## Royal Jelly and Vaginal Atrophy

**Thinning (atrophy) of vaginal tissues after menopause** can lead to discomfort, soreness and painful sex. Ninety postmenopausal women attending a gynaecology clinic with symptoms of vaginal atrophy were treated with either a Royal Jelly vaginal cream (15% strength), vaginal oestrogen cream or a non-hormonal lubricant, for three months.

- **The results showed the vaginal Royal Jelly cream was significantly more effective than the vaginal oestrogen cream or lubricant in improving quality of life.**
- **Improvements in the quality of the vaginal lining cells were also better than in those receiving the prescribed oestrogen cream.**

**Thinning of vaginal tissues can lead to urinary problems** (stress incontinence, painful urination) in postmenopausal women. In these same women,

- **Treatment with vaginal Royal Jelly cream was significantly more effective than conjugated oestrogen cream or lubricant in improvement of quality of life, sexual and urinary problems in postmenopausal women.**

American Institute of Integrative Oncology RESEARCH & EDUCATION



## Vitamin E and D Suppositories and Vaginal Atrophy

Vitamin E Suppository 100 iu. 67 mg d alpha tocopherol

Vitamin D Suppository 1000 iu. 25 mcg

protective effects that decrease the mean pain during intercourse, vaginal pH, dryness, and paleness and help increase the vaginal maturation value

Iran J Nurs Midwifery Res. 2016 Sep-Oct; 21(5): 475-481.
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**The effect of vitamin D on vaginal atrophy in postmenopausal women** Parastou Bad,[1]

American Institute of Integrative Oncology RESEARCH & EDUCATION



## BONE HEALTH
## OSTEOPOROSIS

American Institute of Integrative Oncology RESEARCH & EDUCATION

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## Dr. Nalini's Custom Bone Support Tonic

480 ml

| | | |
|---|---|---|
| 180 | You Gui Tang - | Restore Right Decoction |
| 80 | Bu Gu Zhi | Fr. Psoralea coryfolia |
| 80 | Yin Yang Huo | H. Epimedium |
| 50 | Dan Shen | Rdx Salvia miltiorrhiza |
| 30 | Shan Zhu Yu | Fr. Corni |
| 30 | Bai Shao | Rdx Paeonia alba |
| 30 | Xu Duan | Rdx Dispaci |

ACUPOINTS

K3, K7, CV 4
Sp3, St 36
UB 20, UB 23,
GV3, GV 4

2 teaspoons daily



## OSTEOHERBAL FORMULA
## Health Concerns

Chi Shao
Chuan Xiong
Dang Gui (Shen)
Dang Shen
Gan Cao
Gui Ban
Gui Ban Jiao
Gui Zhi
Ji Xue Teng
Lu Jin
Mu Gua
Rou Cong Rong
Shu Di Huang
Wu Zhu Yu
Zou Ma Tai

Peony (Red)
Ligusticum
Tangkuei
Codonopsis
Licorice
Testudinis
Fresh-Water Turtle Shell
Cinnamon Twigs
Milettia
Deer Ligament
Chaenomeles
Cistanches
Rehmannia (Cooked)
Evodia Fruit
Ardisia

3 tablets daily
PLUS DFH OSTEOBEN 2 caps bid

WARMING
Tonifies Kidney Yang
Invigorates Blood
Supports Normal Growth of Bone Matrix

## High Quality Chinese Herbs Companies and Suppliers

Sun Ten and Brion Herbs
Ming Tong Herbs
TCM Zone Chinese Herbs
Golden Lotus Herbs
Golden Flower Chinese Herbs
Health Concerns Formulas
Evergreen Herbs
Heron Botanicals
Herbal Vitality
Blue Poppy

Distributors
LhasaOMS.com
EmersonEcologics.com
NaturalPartners.com

frontiers in Pharmacology

REVIEW
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# Chinese Medicines Improve Perimenopausal Symptoms Induced by Surgery, Chemoradiotherapy, or Endocrine Treatment for Breast Cancer

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The application of surgery, chemoradiotherapy, and endocrine treatment successfully increases survival rates of breast cancer patients. However, perimenopausal symptoms, the main side effects of these treatments, often afflict patients and reduce their quality of life. Perimenopausal symptoms include vasomotor symptoms, sleep problems, arthromuscular symptoms, and osteoporosis. Currently, there are no satisfactory treatments for perimenopausal symptoms that result from these treatments. Therefore, alternative and complementary therapies including herbal medicines represented by Chinese medicines (CMs), acupuncture, massage, and psychotherapy are increasingly being expected and explored. In this paper, we review the effects and potentials of several CM formulae, along with some active ingredients or fractions from CMs, Chinese herbal extracts, and other herbal medicines, which have drawn attention for improving perimenopausal symptoms in breast cancer patients. We also elaborate their possible mechanisms. Moreover, further studies for evaluation of standardized clinical efficacy should be scientifically well-designed and continuously performed to investigate the efficacy and mechanisms of CMs for perimenopausal symptoms due to breast cancer therapy. The safety and value of estrogen-containing CMs for breast cancer should also be clarified.

**Keywords: Chinese medicine, herbal medicine, complementary therapies, breast cancer, perimenopausal symptoms, quality of life**

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## INTRODUCTION

With the comprehensive applications of surgery, radiotherapy, chemotherapy, and endocrine therapy, more women are becoming long-term breast cancer survivors (Bouzbid et al., 2018). However, the increased survival rate is not always accompanied by an improved quality of life. Many breast cancer survivors are plagued by perimenopausal symptoms caused by these treatments, such as vasomotor symptoms, sleep problems, unhealthy emotions, sexual dysfunction, arthromuscular symptoms, and osteoporosis (Hickey et al., 2008; Jeruss and Woodruff, 2009; Park I. H. et al., 2012). After undergoing bilateral oophorectomy, most patients develop severe and

sustained hot flushes and other menopausal symptoms (Bachmann, 1999). Chemotherapy and pelvic radiotherapy inhibit ovarian function and cause premature menopause, which adversely affect fertility and sexual function in young breast cancer patients and thus cannot be ignored (Azim et al., 2011). These young survivors may also experience secondary problems in their cardiovascular or skeletal systems (Jeruss and Woodruff, 2009). Although the clinical application of endocrine drugs has greatly improved the survival rates of patients with hormone-dependent breast cancer and reduced the risk of recurrence and metastasis (Fisher et al., 2001), 63.7% of patients taking tamoxifen and 72.7% of patients taking the aromatase inhibitor letrozole have been shown to develop cardiovascular and cerebrovascular events or perimenopausal symptoms including hot flushes, night sweats, arthralgia, and myalgia (Breast International Group (BIG) 1-98 Collaborative Group et al., 2005). As many as one-fifth of breast cancer patients consider stopping endocrine therapy because of their menopausal symptoms (Fellowes et al., 2001), which affects treatment adherence and greatly limits efficacy.

Physical discomfort, bad moods, and social embarrassment due to premature menopause, as well as the long-term, repeated menopausal symptoms in breast cancer patients, cause significant decline in quality of life (Schover, 1994; Gracia and Freeman, 2004). Perimenopausal symptoms are among the most common adverse effects of breast cancer treatment in women of various ages. However, at present, few drugs are available that effectively treat perimenopausal symptoms due to breast cancer therapy. Symptomatic treatment is mainly used in clinical practice, including hormone replacement therapy (HRT), selective serotonin reuptake inhibitors (SSRIs), selective serotonin-norepinephrine reuptake inhibitors (SNRIs), vitamin E, and oryzanol. However, these treatments have many problems in their practical applications. HRT is controversial because it may increase the risk of thromboembolic disease, stroke, breast cancer, endometrial cancer, and ovarian cancer (Rossouw et al., 2002; Archer and Oger, 2012; Henderson and Lobo, 2012; Lee et al., 2016; Sjögren et al., 2016). Specifically, HRT is not recommended for patients with hormone-dependent breast cancer, while recent studies have suggested that its safety should be reconsidered (Fahlén et al., 2013). SSRIs and SNRIs have some effects on perimenopausal symptoms of breast cancer but can cause serious adverse effects such as constipation, dry mouth, and decreased appetite, thus limiting their clinical applications (Stearns and Loprinzi, 2003; Sturdee, 2008; Hall et al., 2011). Other treatments, such as vitamin E and oryzanol, either have poor clinical efficacy or remain in the research phase (Barton et al., 1998).

Therefore, with increasing evidence related to their improvement of perimenopausal symptoms associated with breast cancer therapy, alternative, and complementary therapies, such as herbal medicines including Chinese medicines (CMs), acupuncture, massage, and psychotherapy, are attracting increasing attention (Hachul et al., 2014; Lesi et al., 2016; van Driel et al., 2018). CMs and other herbal medicines in particular, are used worldwide to alleviate menopausal symptoms (Hall et al., 2011; Lin et al., 2017; Moore et al., 2017). However, studies are insufficient regarding the existing perimenopausal symptom-related interventions for those with breast cancer, and the efficacy and safety of these interventions remain to be clarified. In this paper, we review the effects and potentials of several CM formulae, along with some active ingredients or fractions from CMs, Chinese herbal extracts, and other herbal medicines (**Table 1**) that have gained attention for improving perimenopausal symptoms due to breast cancer therapy in current clinical and experimental studies. We also elaborate their possible mechanisms to provide a reference for future studies and clinical applications.

# CM FORMULAE

## Shugan Liangxue Decoction

Shugan Liangxue Decoction is a prescription developed by Professor Pingping Li of the Department of Integrated Chinese and Western Medicine of Beijing Cancer Hospital. It mainly comprises *Bupleurum chinense* DC., *Arnebia euchroma* (Royle) Johnst., *Paeonia lactiflora* Pall., *Paeonia suffruticosa* Andr., *Cynanchum atratum* Bge., and *Schisandra chinensis* (Turcz.) Baill (**Table 2**).

Experimental studies have confirmed that Shugan Liangxue Decoction reduced tumor volumes in nude mice with or without ovariectomies. The decoction was also shown to dose-dependently downregulate proliferation of estrogen receptor (ER)-positive breast cancer cells (Fu and Li, 2011; Zhou et al., 2014) and with no significant estrogenic activity (Zhang and Li, 2009; Zhou et al., 2015). Its antitumor activity may be related to its inhibiting key estrogen synthetase, such as aromatase and steroid sulfatase (STS) (Zhang and Li, 2010; Zhou et al., 2014), and may also be related to its selective inhibition of estrogen receptor alpha (ERα) (Zhou et al., 2018). Shugan Liangxue Decoction has no significant influence on the levels of tamoxifen or its metabolites in the human body (Sun and Li, 2009). *In vivo* studies in mice have shown a synergistic effect when Shugan Liangxue Decoction is used with tamoxifen, as it enhances anti-tumor effect of tamoxifen (Wu and Li, 2008) and alleviates tamoxifen's side effects on endometrial thickening (Li et al., 2003). In addition, Shugan Liangxue Decoction combined with anastrozole promotes osteoblast proliferation, enhances osteogenesis (Zhou et al., 2015), and improves bone metabolism (Liu et al., 2009), suggesting that Shugan Liangxue Decoction may improve bone loss caused by endocrine drugs.

Clinical studies have confirmed that Shugan Liangxue Decoction alleviates hot flushes and insomnia in breast cancer patients taking tamoxifen. A randomized, double-blind, placebo-controlled study (Sun et al., 2009) enrolled 73 breast cancer patients (the treatment vs. the control: 37 vs. 36) who developed hot flushes after taking tamoxifen. The patients were continuously treated for 21 days, and the results showed that the proportion of patients in the treatment group whose hot flashes disappeared was 15.2% (vs. 0% in the control group), and the improvement rate was 57.6% (vs. 30.3% in the control group). Further, the proportions of patients with sleep improvement in the treatment and control groups were 63.6 and 39.4%, respectively. All indicators in the treatment

group were significantly better than those in the control group. Serum estradiol levels of patients in the treatment group did not significantly change before or after treatment, and no adverse reactions were noted. A similar study (Xue D. et al., 2011) enrolled and analyzed 60 breast cancer patients receiving adjuvant endocrine therapy, of whom 32 patients received Shugan Liangxue Decoction for 6 months per year for over 2 years in addition to the endocrine therapy, while 28 patients received endocrine therapy alone. Such long-term use of Shugan Liangxue Decoction significantly improved patients' hot flushes and sleep without obvious toxicity. Furthermore, the decoction did not affect tumor recurrence or metastasis.

## Erxian Decoction

Erxian Decoction was created by Professor Berna Zhang of the Shuguang Hospital of Shanghai University of Traditional Chinese Medicine. It consists of *Curculigo orchioides* Gaertn, *Epimedium brevicornum* Maxim., *Morinda officinalis* How, *Phellodendron chinense* Schneid., *Anemarrhena asphodeloides* Bge., and *Angelica sinensis* (Oliv.) Diels (**Table 2**). It is mainly used for menopausal syndrome (Zhong et al., 2013) and is also often used for osteoporosis (Li et al., 2017) and premature ovarian failure (Hu et al., 2013). For decades, Erxian Decoction has been widely used to improve various menopausal symptoms, such as hot flushes, night sweats, insomnia, and depression, due to its definite therapeutic effect with no severe adverse reactions reported (Chen et al., 2008). Recently, network pharmacology studies suggested that about 20 compounds in Erxian Decoction may be the potentially effective ingredients in relieving menopausal symptoms (Wang et al., 2015).

Clinical studies on Erxian Decoction have suggested that it positively affects perimenopausal symptoms in breast cancer patients. One randomized controlled trial (Shao et al., 2015) compared the clinical efficacy of Erxian Decoction combined with tamoxifen vs. tamoxifen alone in treating premenopausal patients with advanced breast cancer (59 cases in each group). The results showed that the total score of CM symptoms, including fatigue, loss of appetite, hot flashes, night sweats, and sleep quality in the Erxian Decoction group were significantly improved after 2 months of treatment. Long-term follow-up also showed that the duration of taking tamoxifen in the Erxian Decoction group was significantly longer than that in the control group. Erxian Decoction can also improve menopausal symptoms, including hot flushes, night sweats, and dysphoria, in breast cancer patients with amenorrhea after postoperative chemotherapy (Liu et al., 2007).

*In vivo* studies showed that Erxian Decoction increased serum estrogen levels by upregulating ovarian aromatase and phosphorylated protein kinase B (p-PKB), thereby alleviating menopausal symptoms (Sze et al., 2009; Wang et al., 2017). *In vitro* studies confirmed that Erxian Decoction could stimulate estrogen production and inhibit proliferation induced by estrogen and metastasis of breast cancer cell as well (Gao et al., 2016; Wang et al., 2017). Erxian Decoction also protected ovaries from chemotherapy injuries (Yuan et al., 2011; Yang et al., 2016) and had less impact on the uterus, mammary gland and vagina of ovariectomized rats (Xue et al., 2012), thus indicating its safety.

**TABLE 1 |** CM formulae, active ingredients or fractions from CMs, Chinese herbal extracts, and other herbal medicines for perimenopausal symptoms in breast cancer.

| | **Name** |
|---|---|
| CM formula | Shugan Liangxue Decoction |
| | Erxian Decoction |
| | Xiaoyao Powder |
| Active ingredient or fraction from CM | Tenuigenin |
| | Resveratrol |
| | Genistein |
| Chinese herbal extract | *Salvia miltiorrhiza* Bge. extract |
| | Ginkgo biloba extract |
| Other herbal medicine | Black cohosh |
| | Red clover |
| | *Humulus lupulus* L. |

**TABLE 2 |** Composition of Chinese medicine formulae.

| **CM formula** | **Composition of CM formula** |
|---|---|
| Shugan Liangxue Decoction | *Bupleurum chinense* DC., *Arnebia euchroma* (Royle) Johnst., *Paeonia lactiflora* Pall., *Paeonia suffruticosa* Andr., *Cynanchum atratum* Bge., and *Schisandra chinensis* (Turcz.) Baill. |
| Erxian Decoction | *Curculigo orchioides* Gaertn, *Epimedium brevicornum* Maxim., *Morinda officinalis* How, *Phellodendron chinense* Schneid., *Anemarrhena asphodeloides* Bge., and *Angelica sinensis* (Oliv.) Diels. |
| Xiaoyao Powder | *Bupleurum chinense* DC., *Angelica sinensis* (Oliv.) Diels, *Paeonia lactiflora* Pall., *Atractylodes macrocephala* Koidz., *Poria cocos* (Schw.) Wolf, *Glycyrrhiza uralensis* Fisch., *Zingiber officinale* Rosc., and *Mentha haplocalyx* Briq. |

In addition, Erxian Decoction reduced levels of serum total cholesterol, low-density lipoprotein cholesterol, and modulated blood lipid levels in postmenopausal rats (Sze et al., 2011). Erxian Decoction could also improve osteoporosis in ovariectomized rats (Xue L. et al., 2011) and regulate osteoblast activity and bone metabolism (Zhu et al., 2010), and its bone protection may be related to the ER-mediated signaling pathway (Wong et al., 2014).

## Xiaoyao Powder

Xiaoyao Powder is derived from the *Prescriptions of the Bureau of Taiping People's Welfare Pharmacy* issued by the government in 1151. It consists of *Bupleurum chinense* DC., A*ngelica sinensis* (Oliv.) Diels, *Paeonia lactiflora* Pall., *Atractylodes macrocephala* Koidz., *Poria cocos* (Schw.) Wolf, *Glycyrrhiza uralensis* Fisch., *Zingiber officinale* Rosc., and *Mentha haplocalyx* Briq (**Table 2**). Depending on the patient's symptoms, in clinical practice, corresponding CMs are added to the Xiaoyao Powder under the guidance of the CM principle for syndrome differentiation and treatment to create the modified Xiaoyao Powder. Xiaoyao Powder and modified Xiaoyao Powder are mainly used to treat menopausal syndrome and premenstrual syndrome (Scheid et al., 2010; Chen H. Y. et al., 2015) and

are frequently used for cancer, insomnia, functional dyspepsia, and poststroke depression (Qin et al., 2009; Bai et al., 2010; Lee K. H. et al., 2013; Tsai et al., 2014; Liao et al., 2016). Clinical applications confirmed that Xiaoyao Powder and modified Xiaoyao Powder effectively improved perimenopausal symptoms, such as insomnia and emotional disorder (Chen et al., 2011; Terauchi et al., 2011; Wang et al., 2014).

A 10-year analysis of 20,466 breast cancer patients treated with tamoxifen showed that more than half the subjects had ever used CMs, in which modified Xiaoyao Powder had the highest utilization rate, nearly one-third. The analysis showed that application of CMs reduced the risk of endometrial cancer induced by tamoxifen (Tsai et al., 2014). What is the effect of modified Xiaoyao Powder on perimenopausal symptoms in breast cancer patients? A randomized controlled trial (Sun and Zhang, 2013) observed modified Xiaoyao Powder's therapeutic effects on breast cancer patients receiving tamoxifen and enrolled 31 patients administered with the modified Xiaoyao Powder and 30 cases with tamoxifen alone. After 2 months of treatment, the Kupperman Index of the modified Xiaoyao Powder group was significantly lower than that of the control group. Meanwhile modified Xiaoyao Powder did not affect the estrogen levels. Other clinical studies (Xu et al., 2005; Zhang and Zheng, 2012; Fu, 2016; Zhao et al., 2017) also showed that modified Xiaoyao Powder produced some improvement of perimenopausal symptoms of breast cancer and had good safety; however, more high-quality studies are needed to support the present research conclusion.

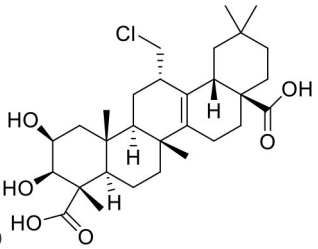
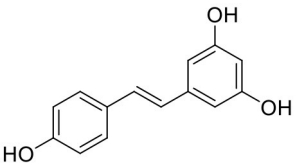
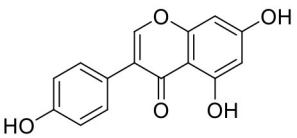
*In vivo* and *in vitro* studies have shown that Xiaoyao Powder induced apoptosis and autophagy of hormone-dependent breast cancer MCF-7 cells (Wang et al., 2009; Li et al., 2016a,b) and inhibited hormone-dependent and hormone-independent growth of breast tumor (Chen et al., 2012; Qi et al., 2015; Li et al., 2016a,b). Studies evaluated estrogen-like effects of flavonoid components in Xiaoyao Powder and found that they enhanced ERα and ERβ expression and promoted MCF-7 cell proliferation (Chen et al., 2016). While some researchers (Song and Li, 2013) confirmed that although *Angelica sinensis* (Oliv.) Diels in Xiaoyao Powder revealed phytoestrogen-like effects, Xiaoyao Powder itself had no effect on tumor growth and did not exhibit estrogen-like effects. Such inconformity also reflects the complexity of the efficacy of CM formulae containing complex mixtures of naturally-occurring chemicals, which needs further exploration and more evidence to confirm the effects of Xiaoyao Powder on estrogen levels and ERs.

# ACTIVE INGREDIENTS OR FRACTIONS FROM CMS

## Tenuigenin

Tenuigenin is one of the main active ingredients of one CM herb, *Polygala tenuifolia* Wild (**Table 3**). Pharmacological studies have shown that tenuigenin plays roles in neuroprotection, memory and cognitive improvement, and antioxidation (Sun et al., 2007; Chen et al., 2010; Liang et al., 2011; Huang et al., 2013). Clinically, the reduction of estrogen, such as after ovariectomy, may lead to cognitive impairment (Walf et al., 2009; Su et al., 2010), and breast cancer patients after ovariectomy may have problems with learning and memory. Studies (Cai et al., 2013) have shown that tenuigenin improved memory and cognitive deficits in ovariectomized mice, which may be related to its reducing the loss of nitric oxide synthase (NOS)-positive neurons and improving the changes in synaptic morphology of the hippocampal CA1 area induced by ovariectomy. This suggests that the therapeutic effects of tenuigenin on menopausal neurological symptoms and cognitive dysfunction after ovariectomy are worth further study.

**TABLE 3 |** Information of active ingredients of Chinese herbal medicine.

| Active ingredient of Chinese herbal medicine | CAS Rn* | Molecular structure of active ingredient |
|---|---|---|
| Tenuigenin (Senegenin) | 667438-01-9 (2469-34-3) |  |
| Resveratrol | 501-36-0 |  |
| Genistein | 446-72-0 |  |

*CAS Rn: Chemical Abstracts Service Registry Number.*

## Resveratrol

Resveratrol is widely present in common plants, such as grapes, peanuts, and *Polygonum cuspidatum* Sieb. et Zucc. (Li T. K. et al., 2016), and has several biological activities including antitumor and anti-cardiovascular disease action, neuroprotection, antioxidation, and liver protection (Liman et al., 2000; Athar et al., 2007; Rivera et al., 2008; Liu et al., 2011) (**Table 3**).

Many studies have been conducted on resveratrol improving menopausal symptoms. A 14-week randomized double-blind placebo-controlled study enrolled 80 postmenopausal women receiving trans-resveratrol (75 mg, twice daily) and suggested that resveratrol relieved the chronic joint pain in menopausal women (Rhx et al., 2017). Other randomized controlled trials found that resveratrol could improve cerebrovascular and cognitive function (Evans et al., 2017), decrease the number of vasomotor symptoms, and alleviate the degree of hot flashes in menopausal women (Leo et al., 2015).

Animal experiments verified that oral intake of resveratrol had less effect on the endometrium (Zhang W. Z. et al., 2008). *In vivo* and *in vitro* studies have also demonstrated that resveratrol has anti-breast cancer effects (Scarlatti et al., 2008; He et al., 2011; Fu et al., 2014). Although resveratrol is expected to improve menopausal symptoms in breast cancer patients, it remains highly controversial because of its possible estrogen-like effect at present. Resveratrol can competitively bind to ERs, and researchers suggest that such estrogen-like effects may be related to its ovarian protection (Banu et al., 2016) and improvement of menopausal symptoms. Studies in the 1990s have found that resveratrol had the anti-estrogen effect and dose-dependent inhibition of the growth of ER-positive breast cancer MCF-7 cells (Lu and Serrero, 1999). However, in additional studies, researchers discovered resveratrol's dual identity as both estrogen agonist and antagonist, which might account for the conflicting results in studies of resveratrol and estrogen-related cancers. Therefore, determining resveratrol's safety in different breast cancer patient subgroups will be the primary task of future clinical research (Bartolacci et al., 2018).

### Genistein

The adverse effects of HRT are mainly related to the activation of estrogen receptor subtype ERα, which has bottlenecked HRT use for menopausal diseases and makes phytoestrogens attract more attention than ever. Soy isoflavones are well-recognized and extensively studied phytoestrogens (Setchell, 1998). In Asian countries, each woman consumes ∼50 mg of isoflavones per day, much higher than that in western countries (Messina et al., 2006). This soy-rich diet for Asian women is considered to play an important role in reducing breast cancer incidence (Tham et al., 1998). Genistein is a major natural soy isoflavone (Sarkar and Li, 2002) (**Table 3**). Many studies have shown that genistein plays anti-breast cancer roles by regulating cell cycles, inhibiting cell proliferation (Pagliacci et al., 1994; Upadhyay et al., 2001), inducing apoptosis (Li et al., 1999b), and inhibiting tumor angiogenesis and metastasis (Li et al., 1999a).

Genistein is also closely associated with the improvement of perimenopausal symptoms. A 12-week, multicenter, randomized, placebo-controlled clinical study examined the effect of genistein on improving symptoms in postmenopausal women. Eighty-four postmenopausal women received placebo treatment (42 cases) or a single 3,002 mg dose of synthetic genistein (40 cases). The results showed that the number and duration of hot flashes in the genistein group were significantly decreased, and no statistical differences were found in 17β-estradiol, follicle stimulating hormone (FSH), endometrial thickness, or adverse events compared with the placebo group (Evans et al., 2011). Genistein also improves postmenopausal osteoporosis, vaginal atrophy-related symptoms, dry eye syndrome, and cardiovascular risk (Crisafulli et al., 2005; Le et al., 2011; Shao et al., 2012; Arcoraci et al., 2017). Despite all this, limited by its identity as a phytoestrogen, genistein's risk and safety for clinical use in the treatment of breast cancer patients are still yet to be fully considered. Genistein not only antagonizes ERα and its mediated signaling pathway (Choi et al., 2014) but also has a stronger affinity for estrogen receptor beta (ERβ) than for ERα (Chang et al., 2008), which is a natural selective estrogen receptor modulator (SERM) (Sareddy and Vadlamudi, 2015). However, some experimental studies (Pons et al., 2016) suggest that the ERα/ERβ ratio should be considered carefully when genistein is used to treat breast cancer patients. Using genistein in patients with a high ERα/ERβ ratio may be counterproductive. Thus, more experimental and clinical studies are needed to verify the effect and safety of genistein for perimenopausal symptoms of breast cancer.

## CHINESE HERBAL EXTRACTS

### *Salvia miltiorrhiza* Bge. Extract

*Salvia miltiorrhiza* Bge. is a CM herb with the function of promoting blood circulation to remove blood stasis, which has a long history of clinical use, mainly for cardiovascular and cerebrovascular diseases but also in liver and kidney diseases (Wang, 2010; Sun et al., 2015). In China, it is also frequently used for menopausal disorders (Guo et al., 2014; Kwok et al., 2014). There are *Salvia miltiorrhiza* Bge. aqueous extracts (mainly containing tanshinol, caffeic acid, salvianolic acid A, and salvianolic acid B) and alcoholic extracts (mainly containing tanshinone I, tanshinone IIA, cryptotanshinone, tanshinlactone, acetyltanshinone IIA, isocryptotanshinone, dihydrotanshinone I, and neo-tanshinlactone) (**Figure 1**). Modern research demonstrates that many compounds of *Salvia miltiorrhiza* Bge. extract display anti-tumor activity (Zhang et al., 2012; Chen et al., 2014; Sung et al., 2015; Shen et al., 2016), which gives promising prospects for the treatment of breast cancer (Yang et al., 2010; Gong et al., 2012; Kim et al., 2017). Furthermore, *Salvia miltiorrhiza* Bge. extract has also been shown to prevent bone loss, reduce serum triglyceride and low-density lipoprotein cholesterol levels (Zhang et al., 2016), and protect vascular function (Li et al., 2013) in ovariectomized rats. Some researchers suggest that *Salvia miltiorrhiza* Bge. might be a potential SERM with a strong affinity for ERβ (Zhang et al., 2016), and its heart and bone protective effects are mediated by ERs (Weng et al., 2013; Xu et al., 2017).

As the representative active ingredient of the *Salvia miltiorrhiza* Bge. extracts, Tanshinone IIA is chosen as the biomarker for quality control of Danshen in the 2010 edition of Chinese Pharmacopeia. Tanshinone IIA exhibited anti-estrogen properties and inhibited the growth of breast cancer cells (Zhao et al., 2015). Many effects of tanshinone IIA, including cardiovascular protection (Xu et al., 2009; Fan et al., 2011), neuroprotection (Shen et al., 2011), and antiosteoporosis (Kwak et al., 2006), are closely related to menopausal problems. Tanshinone I could significantly induce the apoptosis of ER-positive (MCF-7) and ER-negative (MDA-MB-231) cells (Nizamutdinova et al., 2008), and inhibit the growth of breast cancer cells by the downregulation of Aurora A (Gong et al., 2012). It also has neuroprotective effects (Lee J. C. et al., 2013; Jing et al., 2016). Cryptotanshinone could lead to the apoptosis of MCF-7 cells as a potent stimulator of ER stress mediated by mitogen-activated protein kinases

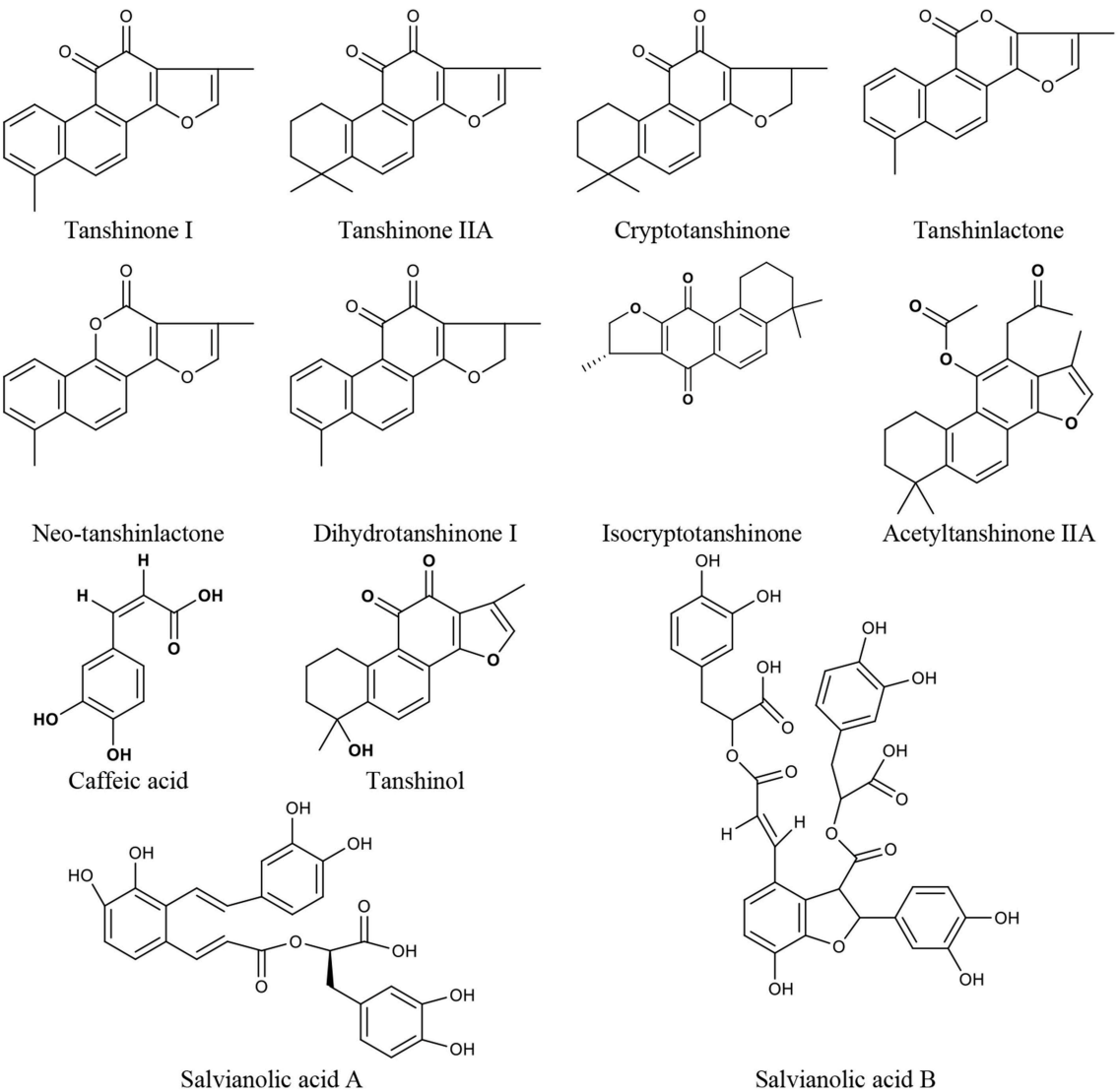
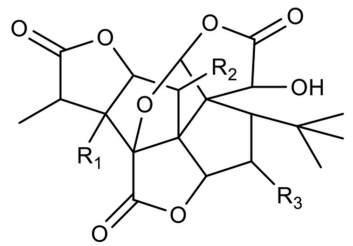



**FIGURE 1 |** The chemical structures of compounds isolated from *Salvia miltiorrhiza* Bunge.

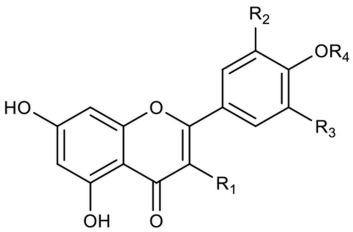
(Park I. J. et al., 2012), and inhibit the growth of ERα-positive breast cancer cells by competitively binding to ERα to suppress ER transcriptional activity (Li et al., 2015). It also possesses cardiovascular protection and neuroprotective effects (Yoo and Park, 2012; Oche et al., 2016). Neo-tanshinlactone inhibited growth and induced apoptosis of ER-positive breast cancer cells through decreasing ERα expression levels and transcriptional activities (Lin et al., 2016). Thus, *Salvia miltiorrhiza* Bge. and its active ingredients might be the potential drugs for treating perimenopausal symptoms due to breast cancer therapy.

## Ginkgo Biloba Extract

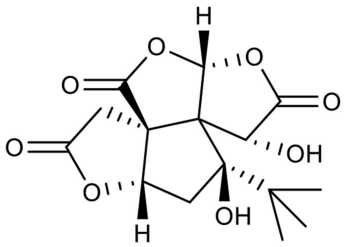
Ginkgo biloba extract (GBE) is a mixture of medicinal ingredients extracted from dried *Ginkgo biloba* L leaves. The predominant pharmacologically active constituents of GBE were identified to be flavonols (quercetin, kaempferol, isorhamnetin, myricetin, apigenin, luteolin, and tamarixetin) and terpene trilactones (ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, ginkgolide M, and bilobalide) (Mohanta et al., 2014) (**Figure 2**). GBE has many effects, such as antioxidation, antiplatelet aggregation, anti-inflammation, and antitumor activity (Packer, 1994; Duttaroy et al., 1999; Ilieva et al., 2004; Dias et al., 2008; Zhang Y. et al., 2008). Clinically, GBE improves menopausal cognitive function (Yuan et al., 2017). A triple-blind, placebo-controlled trial enrolled 80 healthy female volunteers in which 40 individuals received a dose of 120–240 mg GBE while the other 40 received the placebo daily for 30 days. The results showed that GBE positively affected sexual desire in menopausal women (Pebdani et al., 2014). Experimental studies revealed that GBE reduced body weight and adiposity in ovariectomized rats by downregulating 5-HT levels in the hypothalamus (Banin




| | R1 | R2 | R3 |
|---|---|---|---|
| Ginkgolide A | OH | H | H |
| Ginkgolide B | OH | OH | H |
| Ginkgolide C | OH | OH | OH |
| Ginkgolide J | OH | H | OH |
| Ginkgolide M | H | OH | OH |




| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Quercetin | OH | OH | H | H |
| Kaempferol | OH | H | H | H |
| Isorhamnetin | OH | $OCH_3$ | H | H |
| Myricetin | OH | OH | OH | H |
| Apigenin | H | H | H | H |
| Luteolin | H | OH | H | H |
| Tamarixetin | OH | H | OH | $CH_3$ |




Bilobalide

**FIGURE 2 |** The chemical structures of compounds isolated from Ginkgo biloba extract.

et al., 2017), inhibited central neurodegeneration (Shi et al., 2010), significantly increased cognitive function in rats (El Tabaa et al., 2017), and controlled bone loss caused by the lack of estrogen (Trivedi et al., 2009). Ginkgo biloba contains phytoestrogens. Researchers (Oh and Chung, 2004) studied the estrogen activity of ginkgo biloba and its main components (kaempferol, quercetin, and isorhamnetin), and found that these components affected both ERα and ERβ but showed greater affinity for ERβ than ERα, and induced the transcription of pS2 gene and progesterone receptor in MCF-7 cells. Recent studies have shown that GBE restrains estrogen-sensitive breast cancer by inhibiting aromatase and estrogen production (Park et al., 2015, 2016), restrains the proliferation of ER-negative breast cancer cells independently of the ERs (Park et al., 2013; Zhao et al., 2013). Additionally, GBE has a synergistic effect with tamoxifen (Dias et al., 2013).

## OTHER HERBAL MEDICINES

There are also a number of other herbal medicines that have attracted widespread attention for their role in improving perimenopausal symptoms in breast cancer patients.

Black cohosh, a plant that contains many active ingredients such as triterpene glycosides, is one of the most widely used herbs in Europe for relieving menopausal symptoms in women (Reed et al., 2005; Pockaj et al., 2006; Bai et al., 2007). Studies have shown that black cohosh extract improves hot flashes, night sweats, insomnia, anxiety, and other perimenopausal symptoms in breast cancer patients (Vermes et al., 2005; Rostock et al., 2011). It does not increase breast density, endometrial thickness (Hirschberg et al., 2007), or the risk of breast cancer recurrence and metastasis (Henneickevon Zepelin et al., 2007; Obi et al., 2009). Both *in vivo* and *in vitro* studies have indicated that black cohosh extract has no estrogenic activity (And and Henion, 2001; Lupu et al., 2003), while it has demonstrated antiestrogen effects and an inhibiting effect on breast cancer cell proliferation (Bodinet and Freudenstein, 2004; Einbond et al., 2004). There are many theories about its mechanism of action to date. Some scholars believe that black cohosh does not work through ERs but through neurotransmitters such as 5-HT instead (Burdette et al., 2003). It is also hypothesized that the mechanism of action of black cohosh is similar to SERMs according to its clinical estrogen-like effects. However, results of clinical studies on improving menopausal symptoms with black cohosh are sometimes inconsistent, and some studies found its efficacy was not significantly different from placebo treatment (Geller et al., 2009; Fritz et al., 2014; Tanmahasamut et al., 2015). Considering the differences in black cohosh plant types, extracting methods, dosages, and enrolled populations in

different studies, its effectiveness is yet to be explored in larger sample clinical trials. With regard to the adverse reactions of black cohosh, in addition to hepatotoxicity (Mahady et al., 2008), a study found that black cohosh increased the incidence of lung metastasis in c-erbB2-positive transgenic breast cancer mice. It suggests that the safety of long-term use of black cohosh products may need further consideration (Davis et al., 2008).

In addition, red clover (*Trifolium pratense* L.), a perennial plant, is employed in improving menopausal symptoms. Some studies showed that red clover isoflavone extract might improve hot flash frequency (van de Weijer and Barentsen, 2002), while a meta-analysis involving 6 randomized studies did not support the conclusion of red clover reducing vasomotor symptoms (Nelson et al., 2006). As for the safety of red clover for breast cancer, several clinical trials reported that red clover showed no significant effect on estradiol increase or on breast and endometrial thickness in postmenopausal women (Charlotte et al., 2004; Powles et al., 2008). Hops (*Humulus lupulus* L.) are an important ingredient in beer brewing and are used in dietary supplements to improve menopausal symptoms in Europe. Currently, there is still not enough clinical evidence to support the beneficial effect of hops on menopausal syndrome (Palmieri et al., 2009; Erkkola et al., 2010). Meanwhile, studies revealed that both red clover extracts and hops extract showed significant ER competitive binding and estrogen-induced gene activation. The red clover extract had nearly a 9-fold preference for ERα compared with ERβ, while the hops extract preferentially bound to ERβ receptor twice as much than to ERα. Both red clover extracts and hops extracts showed equivalent ERα activities (Overk et al., 2005). To get further convictive conclusion, more work should be done on the efficacy and safety of these herbs for perimenopausal symptoms of breast cancer.

## DISCUSSION

How to improve quality of life is a major challenge in treating long-term breast cancer survivors. Perimenopausal symptoms due to breast cancer therapy are the most common problems that plague patients' daily lives. Whether it is premature menopause in young breast cancer patients (Murthy and Chamberlain, 2012), or the exacerbation of menopausal symptoms in perimenopausal breast cancer patients, physical, psychological, and social problems are commonly and severely affect these patients' quality of life (Harris et al., 2002; Crandall et al., 2004; Gupta et al., 2006).

For breast cancer patients, methods for improving perimenopausal symptoms are limited. Thus, most breast cancer survivors would like to seek help from complementary and alternative. But the relevant information on these therapies is sometimes unreliable or contradictory, and even clinicians often cannot provide clear recommendations (Légaré et al., 2007; Suter et al., 2007). Among these therapies, herbal medicines, especially CMs, are natural and have become the major choice for patients (Moore et al., 2017). In particular, CM is good at taking measures according to the variability of an individual. CMs have a long application history of improving menopausal symptoms in Asian countries, including China, and are receiving more attention worldwide.

However, CMs used to improve natural menopausal symptoms are not always suitable for treating perimenopausal symptoms of breast cancer patients. On one hand, HRT use is controversial for natural menopausal women, and some herbal medicines with estrogen-like effects are also questioned (Lin et al., 2017). CM safety must be the primary concern of researchers for hormone-dependent breast cancer. On the other hand, perimenopausal symptoms of breast cancer patients occur after surgery, radiotherapy, chemotherapy, and endocrine therapy and are closely related to impaired ovarian function and the sudden decline of estrogen levels, but other non-estrogen causes are also considered. Whether or not the mechanism of perimenopausal symptoms due to breast cancer therapy differs from that of natural menopause requires further study. Furthermore, for perimenopausal or postmenopausal women, after the diagnosis and treatment of breast cancer, the internal mechanisms of the occurrence and exacerbation of their menopausal symptoms are even more complicated. The complexity and diversity of perimenopausal symptoms make it difficult for single-target drugs to solve all these problems. Thus, the multi-targeted and comprehensive effects of CM formulae present advantages. In clinical practice, especially in China, CM formulae have been widely used to improve perimenopausal symptoms in breast cancer patients, and some positive conclusions have been drawn from existing studies. However, the existing clinical research has many problems that may affect the credibility of conclusions. For example, many studies are of limited quantity and quality, and multicenter and large-scale clinical trials are insufficient. Most studies evaluate the curative effect by the total symptom score, while there is a lack of standardized measurements for a single major symptom. Researchers pay more attention to symptoms such as hot flashes, night sweats, and insomnia rather than emotion and sexual function. The research quality heterogeneity, including the different preparation methods of CM formulae, affects the results' comparability and reliability. The time of research observation and follow-up is often too short and there are few long-term safety indicators related to breast cancer recurrence and metastasis. Furthermore, except for the antitumor effects of CM formulae, there is not enough experimental research on aspects such as its estrogenic or anti-estrogenic activity, impact on the ERs statuses, mammary glands and uterine tissue and interactions with other drugs. CMs are considered to be multi-targeted possibly due to the variety of ingredients that they contain, which is one of the most significant characteristics of CMs. Although not all targets of each CM are identified, many targets of CMs, especially some commonly used ones, have been studied and identified. Due to the growing needs from patients suffering perimenopausal symptoms of breast cancer, further work should be done to find the molecular mechanisms of these formulae for their better use in clinical practice. In addition, there are few classic traditional formulae for perimenopausal symptoms of breast cancer studied so far. The classic formulae are the treasure of traditional Chinese medicine, some of which have long been used for treating

female menopausal symptoms. Such classic formulae might be potential research topics of improving menopausal symptoms of breast cancer, which will certainly attract more attention in future. Therefore, extensive applications of CM formulae in perimenopausal symptoms in breast cancer patients still need more supportive data from higher quality, transparent, and in-depth studies.

Many CMs commonly used for menopause contain phytoestrogens. Phytoestrogens are similar to endogenous estradiol in structure and can bind to ER to exert estrogenic or anti-estrogenic effects. The major groups of phytoestrogens include isoflavones, coumarins, lignans, and stilbenes (Basu and Maier, 2018), which show different active effects. Both *Psoralea corylifolia* L. and *Cuscuta chinensis* Lam. are Chinese medicines and are often used in the treatment of osteoporosis (Donnapee et al., 2014; Zhang et al., 2016). It was reported that (Xin et al., 2010) the two coumarins in the EtOH extract of *Psoralea corylifolia* L., isopsoralen and psoralen, were selective activators of ERα, which could significantly promote the proliferation of MCF-7 cells. The four flavonoids, isobavachalcone, bavachin, corylifol A, and neobavaisoflavone, could simultaneously activate both ERα and ERβ. All these compounds could exert estrogenic activities through ER, but they may have different biological effects. Yang et al. studied the antiosteoporosis activity of flavonoids in the crude ethanolic extract of *Cuscuta chinensis* Lam. (Yang et al., 2011). It was revealed that kaempferol and hyperoside significantly increased ALP activity in UMR-106 cells and astragalin promoted the proliferation of UMR-106 cells, which showed estrogenic activity. Quercetin and kaempferol showed potent ER antagonist activity by activating ERα/β-mediated AP-1 reporter expression. It was further suggested that the antiosteoporosis effect of *Cuscuta chinensis* Lam. might be closely related to the estrogenic or anti-estrogenic activities of flavonoids (**Figure 3**). Therefore, phytoestrogens are an important issue when it comes to treating perimenopausal symptoms of breast cancer with CM and other herbal medicines. At present, there are more studies conducted on treating menopausal symptoms with herbs and foods containing phytoestrogens, and many studies have obtained positive results (Bedell et al., 2014; Chen M. N. et al., 2015). However, since phytoestrogens may have similar effects to human endogenous estrogen, as well as the limitation of ethics, data from relevant clinical research on perimenopausal symptoms in breast cancer patients are inadequate. Despite the basic structure of phytoestrogens being similar to that of estradiol, which indicates their estrogen-like properties,

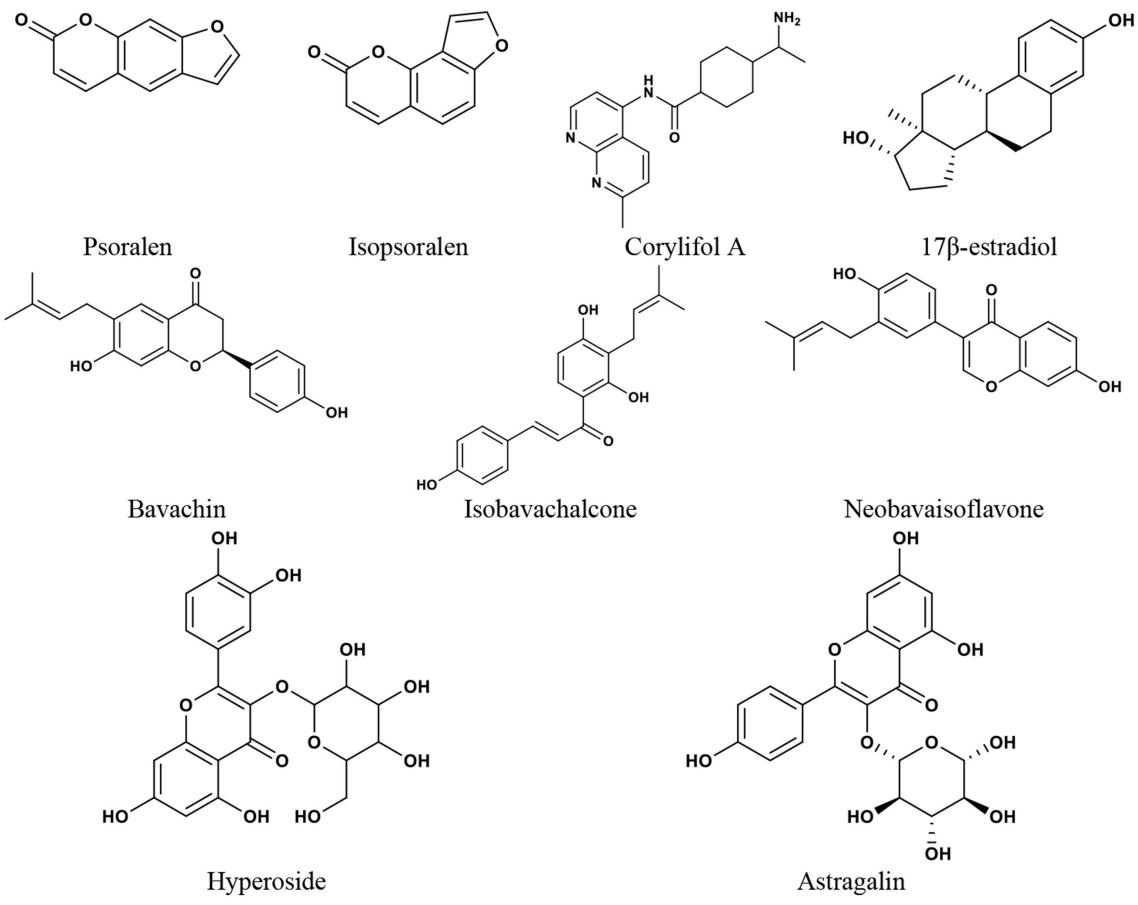



**FIGURE 3 |** The chemical structures of phytoestrogens and 17β-estradiol reported in the discussion section.

phytoestrogen differs from estradiol. Human endogenous estrogen acts mainly via ERα- and ERβ-mediated transcriptional activation or effects (Hillisch et al., 2004). While protecting the cardiovascular, cerebrovascular, nervous, and skeletal systems, human endogenous estrogen has a carcinogenic risk to breasts and the uterus. ERα and ERβ are distributed differently in different tissues. The mammary glands mainly contain ERα, and ERα overactivation is an important factor in the occurrence and development of hormone-dependent breast cancer. As a tissue-specific tumor inhibitor, ERβ has an antiproliferative effect (Nilsson and Gustafsson, 2011). ERβ opposes the effect of ERα by modulating the expression of ERα-regulated genes (Clarke, 2003). Different phytoestrogens have different effects on the two ER subtypes. Studies (Sareddy and Vadlamudi, 2015) have shown that some phytoestrogens exhibit the characteristics of SERMs, which can antagonize ERα or have a higher affinity for ERβ, and can selectively activate the ERβ transcriptional pathway. It may avoid the drawbacks of endogenous estrogen. Genistein interacts with both ERα and ERβ, and has a higher affinity for ERβ (Chang et al., 2008). The affinity of genistein for ERα was 4%, while it was 87% for ERβ, compared with estradiol (Kuiper et al., 1998). Genistein was reported to recruit the steroid receptor coactivator 3 (SRC3) much more efficiently to ERβ than to ERα (Jiang et al., 2013). Meanwhile, genistein could inhibit the expression of ERα, antagonize the signal pathway of ERα, and affect the proliferation and apoptosis of breast cancer cells (Choi et al., 2014). It was also reported that genistein significantly reduced the expression of ERα mRNA and increased the ERβ level in three different ER-positive breast cancer cells, MCF-7, T47D, and 21 PT (Marik et al., 2011). Due to the similarity in structure to the synthetic estrogen diethylstilbestrol, resveratrol interacts with ER and has been designated as the "phytoestrogen" (Mueller et al., 2004). However, resveratrol may be a combination of the agonist and antagonist to estrogen, depending on the dosage and concentration of resveratrol and 17β-estradiol (E2), as well as the expression of ERα and ERβ in tissue cells (Bhat et al., 2001). *Salvia miltiorrhiza* Bge. extract could interact with ERα and ERβ, and significantly induce the expression of ERα/β-estrogen response element (ERE) luciferase reporter gene without side effects on reproductive tissues (Xu et al., 2017). There are many flavonoids in ginkgo biloba extract, which have been demonstrated to affect ERα and ERβ and show a higher affinity for ERβ than for ERα. It can also induce progesterone receptor transcription in MCF-7 cells (Oh and Chung, 2004). In addition to the regulatory effect on estrogen receptor expression, phytoestrogens also inhibit estrogen biosynthesizing enzymes. Promoters I.3 and II are the major promoters directing aromatase expression in breast cancer, and genistein may inhibit the activities of promoters I.3 and II for CYP19 regulation (Chen et al., 1999). A study showed that a methoxy derivative of resveratrol, 3MS, could efficiently inhibit the expression of aromatase protein encoded by CYP19 in MDA-MB- 231 cells (Licznerska et al., 2017). The standard GBE (EGb 761) significantly inhibited aromatase activity and reduced the expression of CYP19 mRNA and CYP19 promoter I.3 and PII (Park et al., 2016). Furthermore, phytoestrogens may inhibit estrogen metabolic enzymes. Genistein impacts the formation of estrogen metabolites and is a potent inhibitor of E1 and E2 sulfation (Poschner et al., 2017). Cytochrome P450 CYP1 family enzymes such as CYP1A1, CYP1A2, and CYP1B1 are important enzymes in estrogen metabolism. It was reported that resveratrol could inhibit dioxin-induced CYP1A1 and CYP1B1 expression levels and recruitment of AHR and ERα in T-47D cells (Macpherson and Matthews, 2010). Meanwhile, many studies have shown that phytoestrogen intake reduces the risk of breast cancer, which may be related to its effects of decreasing estrogen and progesterone levels and reducing endogenous hormonal stimulation in breast tissue (Kumar et al., 2002; Peeters et al., 2004; Touillaud et al., 2005). *In vivo* and *in vitro* studies have also found that some phytoestrogens inhibit breast cancer growth (Li L. et al., 2016). Thus, phytoestrogens with such characteristics would be of great value in treating perimenopausal symptoms due to breast cancer therapy.

Therefore, in this paper, we reviewed the potential roles of CMs in treating perimenopausal symptoms in breast cancer patients, focusing on several CM formulae, along with some active ingredients or fractions from CMs, Chinese herbal extracts and other herbal medicines. We also elaborate their interactions with ERs and anti-breast cancer properties. CM is a kind of medicine originating from the experience of application in humans and has benefitted mankind throughout history. It should be noted that more work on the molecular mechanisms and in-depth pre-clinical studies of CMs are necessary, which will be helpful to explain the biological activity and mechanism, confirm the safety and effectiveness, and determine the ideal dosages for clinical trials and finally for the better use in patients. It is expected that future research will thoroughly investigate the efficacy, effective dose, and adverse reactions of these drugs and their interactions with chemotherapeutics or endocrine drugs, clarify the safety and value of estrogen-containing CMs for breast cancer patients, and screen out drugs with high safety and efficacy in treating perimenopausal symptoms of breast cancer for better clinical use. CMs have been used in clinical practice for a long time and are a potential medicinal source for treating complex diseases. In-depth exploration of the roles, mechanisms and material bases of CMs for perimenopausal symptoms after surgery, radiotherapy, chemotherapy, and endocrine therapy for breast cancer, may provide more choices for patients.

## AUTHOR CONTRIBUTIONS

SW and WC wrote the manuscript. HL and WC designed and edited the manuscript. All authors contributed to and approved the final version of the manuscript.

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# Strategies to promote abundance of *Akkermansia muciniphila*, an emerging probiotics in the gut, evidence from dietary intervention studies

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## Abstract

*Akkermansia muciniphila* is a mucin-degrading bacterium commonly found in human gut. *A. muciniphila* has been inversely associated with obesity, diabetes, inflammation, and metabolic disorders. Due to its highly promising probiotic activities against obesity and diabetes, *A. muciniphila* has drawn intensive interest for research and development in recent years. A number of human and animal studies have shown that the abundance of *A. muciniphila* in the gut can be enhanced through dietary interventions. The present review focuses on evidence-based dietary strategies of improving *A. muciniphila* abundance in the gut by critically appraising up-to-date available human and animal intervention studies on *A. muciniphila* growth and their impact on risk factors of obesity and diabetes. Their potential mechanisms in promoting *A. muciniphila* are also discussed along with the discussions of mechanism of action for *A. muciniphila* to exert probiotic functions.

### Keywords

Probiotics; *Akkermansia muciniphila*; Dietary supplementation; Gut microbiota; Obesity; Diabetes

## 1. Introduction

*Akkermansia muciniphila* is a mucin-degrading bacterium belonging to the phylum *Verrucomicrobia* (Derrien, Collado, Ben-Amor, Salminen, & de Vos, 2008). *A. muciniphila* was first isolated and identified when using purified mucin as the only carbon source in the growing medium in a belief that specific gut microbes have ability to utilize the mucus glycans as carbon sources (Derrien, Vaughan, Plugge, & de Vos, 2004). *A. muciniphila* is commonly found in human gut, representing 3–5% of the microbial community in humans (Belzer & de Vos, 2012; Derrien et al., 2004). The bacterium is also found in a wide variety of other species partly due to its mucin-degrading capability which provides an ecological advantage, especially in a condition of lacking other dietary sources except mucin (as the only constant source of nutrients) (Lukovac et al., 2014). Since its discovery in 2004 by Derrien et al. (2004), *A. muciniphila* has quickly become a popular research topic due to its newly discovered probiotic properties (Derrien et al., 2008; Dingemanse et al., 2015; van Passel et al., 2011). The bacterium is more abundant in the gut of healthy subjects than in that of diabetic and obese patients (Karlsson et al., 2012; Santacruz et al., 2010; Tilg & Moschen, 2014) and patients with bowel diseases (Png et al., 2010) and metabolic disorders

(Brahe et al., 2015; Collado, Isolauri, Laitinen, & Salminen, 2010). Recent intervention studies also confirmed an inverse correlation of *A. muciniphila* abundance with body weight (Everard et al., 2013; Shin et al., 2014), inflammation (Hansen et al., 2014), metabolic syndrome (Roopchand et al., 2015), and both type 1 diabetes (Hansen et al., 2012a, 2012b) and type 2 diabetes (Hansen et al., 2012a, 2012b; Shin et al., 2014). Collectively, the increasing body of evidence from animal and human studies suggest that *A. muciniphila* is a highly promising probiotic, especially its potential for the prevention and treatment of diabetes, obesity, and their associated metabolic disorders, which is of great interest for future research and development.

The exact mechanisms by which *A. muciniphila* exerts the beneficial impact on health have not been fully elucidated. The positive modulation of mucus thickness and gut barrier integrity by *A. muciniphila* could be the key for its aforementioned probiotic activities. *A. muciniphila* supplementation was able to restore mucus thickness in obese and type 2 diabetic mice where gut mucus was disrupted by high fat diet treatment; the treatment also resulted in a significant reduction of serum lipopolysaccharides (LPS), a metabolic endotoxemia, and improved the metabolic profile (Everard et al., 2013). LPS is a major component of the outer membrane of gut gram-negative bacteria and its presence in circulation often indicates gut permeability, thus a disruption of intestinal mucus (Turner, 2009). Intestinal mucus is synthesized and secreted by the goblet cells consisting of two layers: an inner unstirred layer devoid of bacteria and a thicker multi-laminated outer layer with commensal bacteria (Johansson et al., 2008). Its major components, mucins which contain approximately 20% protein and 80% carbohydrates are a source of nutrients for *A. muciniphila* (Ambort et al., 2012). The intestinal adherent mucus gel layers in humans has a thickness from around 200 μM up to 800–900 μM depending partly on the sites (Derrien et al., 2010; Pullan et al., 1994). The mucus creates a protective barrier that prevents noxious agents, destructive hydrolases, and microorganisms from directly contacting the epithelial cell layer (Pullan et al., 1994; Turner, 2009). Mucin has a turnover rate of 6–12 h with the inner layer at a rate of about 1 h and its secretion is believed to be regulated by neural, hormonal, and paracrine effects (Ambort et al., 2012; Linden, Sutton, Karlsson, Korolik, & McGuckin, 2008; Plaisancié et al., 1998). Recent studies showed that *A. muciniphila*, despite its utilization of mucin as a source of nutrients, actually were positively associated with the mucus thickness and intestinal barrier integrity in humans and animals (Collado, Derrien, Isolauri, de Vos, & Salminen, 2007; Everard et al., 2013; Ganesh, 2013; Zhong, 2015). How *A. muciniphila* could promote mucus thickness is not known. One of the reasons could be *A. muciniphila* stimulates mucus turnover rate by making short-chain fatty acids from the degraded mucin, the preferable energy sources for the host epithelium which synthesize and secret mucin. Indeed, *A. muciniphila* supplementation increased the number of mucin-producing goblet cells in mice (Shin et al., 2014).

Research has shown that the abundance of *A. muciniphila* was 3300-fold lower in leptin-deficient obese (ob/ob) mice than in their lean littermates (Everard et al., 2013). The bacterium has also been negatively associated with energy extraction from diet in cold condition and caloric restriction (Chevalier et al., 2015; Dao et al., 2016). The beneficial functions of *A. muciniphila* and its potential mechanisms have recently discussed by Derrien, Belzer, and de Vos (2016). Here we thoroughly examined all up-to-date available

intervention studies on *A. muciniphila* in attempting to identify evidence and practical dietary strategies to promote the growth of *A. muciniphila*, an emerging probiotic in the gut.

## 2. Supplementation with viable *A. muciniphila*: effective and consistent from animal studies

Despite the factor that *A. muciniphila* has been discovered for more than 10 years, there was no human study available on direct dietary supplementation with *A. muciniphila.* Nevertheless, three mice intervention studies have been reported. All of them showed a significant increase of *A. muciniphila* in the gut and/or feces of the recipient mice (two different mice models) (Everard et al., 2013; Li, Lin, Vanhoutte, Woo, & Xu, 2016; Shin et al., 2014). A Western high-fat diet on Apoe−/−mice for 8 weeks reduced the abundance of *A. muciniphila* from around $7.0 \times 10^9$/g feces to $4.6 \times 10^9$/g feces, an over 100-fold decrease. While, supplementation with *A. muciniphila* ($5 \times 10^9$ cfu for 8 weeks) on the Western diet was able to restore the abundance of *A. muciniphila* back to $8.0 \times 10^9$/g feces (Li et al., 2016). Interestingly, supplementation of *A. muciniphila* did not significantly change in the composition of gut microbiota (except the bacterium itself). The *A. muciniphila* supplementation reduced the size of atherosclerotic plaques and ameliorated both aortic and systemic inflammation in Western diet-fed Apoe−/−mice (Li et al., 2016). The other two studies used diet-induced obese (Power et al.) mice. Oral treatment of *A. muciniphila* ($2 \times 10^8$ cfu for 4 weeks) increased *A. muciniphila* abundance in the fecal content of the high-fat-fed DIO mice (from about $10^8$/g feces in the high-fat only-fed group to $10^{10}$/g feces in the treatment group) (Everard et al., 2013). Again, the high-fat diet significantly changed the gut microbiota whereas *A. muciniphila* treatment did not modify this profile (Everard et al., 2013). Another study also found that oral supplementation of *A. muciniphila* ($4.0 \times 10^8$ cfu/d for 6 weeks) was able to restore its abundance in high-fat diet-fed DIO Mice, which was significantly reduced after 4 weeks of HFD (Shin et al., 2014). Collectively, all three studies showed that Western high-fat diet in as short as 4 weeks were able to significantly reduce the abundance of *A. muciniphila* and supplementation of viable *A. muciniphila* is able to restore its abundance back.

Dietary supplementation of *A. muciniphila* did not change microbiota profile (Everard et al., 2013; Li et al., 2016), suggesting a minimum interaction with other gut bacteria. However, other studies found that abundance of *A. muciniphila* was positively associated with the richness of *Gordonibacter*, *Ruminococcaceae*, and *Methanobrevibacter smithii* (Arumugam et al., 2011; Dao et al., 2016). No further report on their interactions or investigation on possible cross-feeding are available. Interestingly, supplementation of *A. muciniphila* did not increase its abundance in DIO mice on normal control diet (both groups at around $10^{10}$/g feces) (Everard et al., 2013), indicating that there could be a up limit for the growth of *A. muciniphila* in the gut and *A. muciniphila* supplementation may not be deliverable for healthy subjects.

These studies also suggest a causal role of intestinal barrier function and LPS in mediating *A. muciniphila* activities (Everard et al., 2013; Li et al., 2016). Bacterial-derived LPS play an essential role in the inflammatory process of inflammatory bowel diseases (IBD) and

other metabolic disorders (Cani et al., 2008; de La Serre et al., 2010; Yu, Flynn, Turner, & Buret, 2005). An increase of serum LPS often suggests an intestinal barrier dysfunction or a leaking gut barrier (Turner, 2009). Thickness of the mucin layer is an important measure of intestinal permeability (Atuma, Strugala, Allen, & Holm, 2001). Research showed that a high-fat-diet (60% fat for 4 weeks) resulted in a 46% thinner mucus layer in DIO mice (Everard et al., 2013). Despite that *A. muciniphila* has been known as a mucin-degrading bacterium (Collado et al., 2007), the bacterium actually increased the thickness of the mucin layer (Li et al., 2016), resulting in a decrease in intestinal permeability and subsequently reduced gut derived LPS penetrating into circulation in Western diet-fed Apoe−/−mice (Li et al., 2016). More interestingly, chronic infusion of LPS was able to complete abolish the benefits of *A. muciniphila* supplementation (the amelioration of inflammation and atherosclerosis), strongly suggesting a causal role of LPS in mediating *A. muciniphila* activities (Li et al., 2016) which could be the result of the reduced intestinal permeability upon *A. muciniphila* supplementation. The LPS-reducing ability (by up to 50% reduction in serum) of *A. muciniphila* supplementation was also confirmed in DIO mice model (Everard et al., 2013; Shin et al., 2014), which could be due to its ability to restore the thickness of the mucin layer and gut barrier function once were damaged by high-fat diet. Although *A. muciniphila* supplementation showed no significant effects on body weight, lipid and glucose metabolism on Apoe−/−mice (Li et al., 2016), it indeed reduced body weight gain, hyperglycemia, insulin resistance on high-fat diet treated DIO mice (Everard et al., 2013; Shin et al., 2014). It is important to note that oral treatment with a lower dose ($4.0 \times 10^6$ cfu) of viable *A. muciniphila* did not ameliorate the impaired glucose tolerance in high-fat-fed DIO mice, suggesting that there is a dose response limit for *A. muciniphila* exert its beneficial effects (Shin et al., 2014).

## 3. Supplement with other selected probiotics promoted *A. muciniphila* growth in the gut

There was an animal study found that oral administration of a mixture of *Lactobacillus rhamnosus* LMG S-28148 and *Bifidobacterium animalis* subsp. *lactis* LMG P-28149 for 14 weeks (5 days/week, $5 \times 10^8$ CFU of each strain in PBS) increased *A. muciniphila* abundance in the fecal content of the high-fat-fed DIO mice by approximately 100 fold (from $10^{6.5}$/g feces on high-fat-fed group to $10^{8.5}$/g feces in high-fat plus probiotics group) (Alard et al., 2016). In this study, a significant inverse correlation was detected between the body weight gain and the abundance of *A. muciniphila* ($P < 0.001$) which is consistent to other intervention studies (Everard et al., 2013; Shin et al., 2014). The effectiveness of this mix probiotics on *A. muciniphila* abundance is surprisingly comparable to that of the direct *A. muciniphila* supplementation on the same mice model (Everard et al., 2013; Shin et al., 2014). It is likely the strain of *B. animalis* subsp. *lactis* LMG P-28149 not *L. rhamnosus* LMG S-28148 exert such an *A. muciniphila*-promoting effect since the authors further conducted a 7-week feeding study on individual strains ($10^9$ CFU each) with the same protocol and found that the benefits of the mix probiotics was attributed to *B. animalis* not *L. rhamnosus* (Alard et al., 2016), though the mechanisms are unclear. Interestingly, previous studies showed that *A. muciniphila* supplementation did not change the gut microbiota profile in DIO mice and Apoe−/−mice (Li et al., 2016), suggesting that *A. muciniphila* does

not directly interact with other gut bacteria. The authors also used a dynamic *in vitro* gut model showing that the mix probiotics induced a significant time-dependent increase of total SCFAs in the simulated ascending colon as well as a shift from acetate to butyrate and propionate. These observations suggest that an increase of SCFAs may be a promoting effect for *A. muciniphila* since SCFAs promote mucin growth and *A. muciniphila* is a mucin-degrading bacterium.

## 4. Supplementation of prebiotics, fructo-oligosaccharides promoted *A. muciniphila* abundance

Although no human study has been reported, three animal studies consistently showed that oral administration of fructo-oligosaccharides (oligofructose or FOS), a common prebiotic, promoted the growth of *A. muciniphila* in the gut of DIO and *ob/ob* mice and Sprague–Dawley rats models (Everard et al., 2011, 2013; Reid, Eller, Nettleton, & Reimer, 2015). A high-fat diet (60% fat for 8 weeks) on DIO mice led to a 100-fold decrease of *A. muciniphila* in feces (from $10^9$/g feces on a standard diet to $10^7$/g feces on high-fat diet), however, prebiotics supplementation (FOS, 0.3 g/d with the high-fat diet for 8 weeks) completely restored its concentration back to the level comparable to the mice fed with a standard diet ($10^9$/g feces) (Everard et al., 2013). The prebiotic effect of FOS on *A. muciniphila* was even more significant on *ob/ob* mice, prebiotic supplementation (FOS, 0.3 g/d with a standard diet for 5 weeks) increased the bacterium abundance by approximately 1000-fold ($10^7$/g feces in the control group versus $10^{10}$/g feces in the prebiotic group) (Everard et al., 2013). Another study found that 5 weeks of oral FOS administration at the same dose increased the abundance of *A. muciniphila* in *ob/ob* mice by over 80 folds, along with the higher abundance of *Bifidobacterium* spp. and the *E. rectale/C. coccoides* group (Everard et al., 2011). In a recent study on newborn male Sprague–Dawley rats, FOS supplementation (10 % in diet for 16 weeks) increased *A. muciniphila* abundance by 2–3 folds without affecting food intake and body weight but showing a trend of increasing glucose metabolism (Reid et al., 2015).

It appeared that prebiotic effect on body weight was inconsistent in different animal models (Everard et al., 2011, 2013; Reid et al., 2015). However, in mice studies, FOS supplementation significantly reduced the total fat mass accompanied by a significant reduction in serum LPS level (by over 50%) and a significant improvement in glycemic control (Everard et al., 2011, 2013). Unfortunately, so far only FOS has been evaluated for such an effect and it is not known whether other prebiotics such as inulin, galacto-oligosaccharides (GOS), lactulose, etc., which all possess similar *A. muciniphila*-promoting activities. It is also unclear how FOS could promote the growth of *A. muciniphila in vivo* and the potential mechanisms have not been discussed by the previous authors. There are also currently no published *in vitro* culture data on *A. muciniphila* utilization of prebiotics including FOS. *A. muciniphila* was unable to utilize specific carbon sources such as glucose, cellobiose, lactose, galactose, xylose, fucose, rhamnose, maltose upon *in vitro* incubation (Derrien et al., 2004). However, based on its genome data, *A. muciniphila* appears capable of metabolizing a variety of carbon sources previously found non-utilizable including galactose, cellobiose, melibiose and fructose (Derrien et al., 2004; van Passel et al., 2011).

Our preliminary *in vitro* culture experiments showed that, among 16 different carbon sources including prebiotics and dietary fibers (FOS, xylooligosaccharide, inulin, galacto-oligosaccharides, isomalto oligosaccharides Karaya gum, potato starch unmodified, methylcellulose fiber, D(+)-raffinose pentahydrate, tragacanth gum, grapefruit pectin, Acacia senegal tummy fiber, beta glucan, psyllium husk, oligo-chitosan, and galactomune), adding FOS into the media significantly promoted *A. muciniphila* growth but other fibers/prebiotics did not exhibited such a strong growth-promoting activity, suggesting FOS may be a preferable nutrient for *A. muciniphila*.

## 5. FODMAP in diet promoted *A. muciniphila*: two human studies suggest A positive association

'FODMAP' refers to fermentable Oligo-, Di- and Mono-saccharides and Polyols—which includes fructose, lactose, oligosaccharides, polyols, and sugar alcohols (polyols, such as sorbitol, mannitol, xylitol and maltitol) which share some distinct functional properties: poor absorption in the small intestine due to lack of hydrolases (fructans, galactans) or limitation of transport across the epithelium (fructose) and rapid fermentation due to their short-chain nature (lactose and oligosaccharides) as compared with dietary fibers (Gibson & Shepherd, 2005, 2010). The ingestion of FODMAPs could increase rapidly-fermentable carbohydrates and subsequently result in functional gut symptoms (Gibson & Shepherd, 2010). For this reason, low FODMAP diets have been widely applied to treat irritable bowel syndrome (IBS) (Halmos, Power, Shepherd, Gibson, & Muir, 2014). However, there currently no clearly defined cut-off values differentiating high or low FODMAP diets. FODMAPs are usually calculated based on individual foods with fructose and fructans being the most widespread in the diet (Gibson & Shepherd, 2010). Recent human studies showed that FODMAP content in diet might significantly affect *A. muciniphila* abundance (Halmos et al., 2015, 2016). A cross over study on 7 patients with Crohn's disease revealed that the patients receiving 21 days of low (containing 3.05% FODMAP) or typical (''Australian" containing 23.7% FODMAP) had *A. muciniphila* abundance of 3.75 or 5.08 ($Log_{10}$ copies of 16S rRNA gene/g feces), respectively, FODMAP diets with 21-day washout in between (Halmos et al., 2016). It should be noted that the low FODMAP diet also significantly lowered the relative abundance of butyrate-producing *Clostridium cluster XIVa*, however, SCFA, pH and total bacterial abundance remained unaltered (Gibson & Shepherd, 2005).

The same research group also conducted a second dietary FOD-MAP intervention on different subjects in a cross-over design: 27 IBS and 6 healthy subjects whom were randomly allocated one of two 21-day provided diets, differing only in FODMAP content (low 3.05 g/day vs Australian 23.7 g/day), and then crossed over to the other diet with ≥21-day washout period (Halmos et al., 2015). Australian diet (high FODMAP) had the relative abundance of *A. muciniphila* at 0.1% versus 0.02% (low FODMAP diet) of total bacteria, their absolute abundance were 5.46 and 4.29 $Log_{10}$ copies of 16S rRNA gene/g feces, respectively (Halmos et al., 2015). The high FODMAP diet also significantly increased the abundance of butyrate-producing *Clostridium cluster XIVa* (Halmos et al., 2015). However, since the authors pooled the analysis together for all subjects (Halmos et al., 2015), it is not known whether FODMAP diets affected the IBS patients and healthy subjects differently

since this study was not aimed to compare fecal microbiota and biochemical indices in healthy subjects with those who have IBS. Contrast to the same intervention on Crohn patients, for IBS patients and health subjects, the low FODMAP diet was associated with higher fecal pH, greater microbial diversity and reduced total bacterial abundance (by 47%) compared with the Australian diet. Nevertheless, fecal SCFA concentration was unaffected (Halmos et al., 2015). It also should be noted that the same research group found that in the same cross-over study protocol on 30 patients with IBS, a diet low in FODMAP effectively reduced functional gastrointestinal symptoms (Halmos et al., 2014). Collectively, the two human studies found that a positive association of FODMAP in diets with *A. muciniphila* abundance in different patients/health subjects. FODMAP diets contained 1.57 and 5.49 Oligosaccharides, respectively (Halmos et al., 2015, 2016), however, it is unclear whether the change of *A. muciniphila* induced by low versus high FODMAP diets are attributable to their difference in oligosaccharide content. Also, unfortunately, there is no human study available investigating the role of FODMAP diet on *A. muciniphila* abundance in obese/diabetic subjects who are associated with low *A. muciniphila* abundance (Everard et al., 2013; Shin et al., 2014).

## 6. Evidence from dietary polyphenol: inconsistent results from human and animal studies

Dietary polyphenols are natural antioxidants and many of them such as phenolic acids, flavones, and anthocyanins possess strong antimicrobial activity (Daglia, 2012; Parkar, Stevenson, & Skinner, 2008). Both antioxidant and antimicrobial activities of dietary polyphenols could potentially reshape the gut microbiota ecology because, on the one hand, many gut bacteria such as *A. muciniphila* are obligate anaerobes, which are extremely vulnerable under the attacks of free oxygen radicals (Daglia, 2012); therefore, dietary antioxidants once ingested may help protect those obligate anaerobes and modify gut microbiota by scavenging oxygen radicals (Roopchand et al., 2015). On the other hand, certain dietary polyphenols have antimicrobial activity against specific bacteria while other bacteria could be promoted. For instance, a recent study showed that a black tea or a red wine grape extract (RWGE), both containing complex dietary polyphenol mixtures, significantly promoted growth of *A. muciniphila* in an *in vitro* gut microbial ecosystem, namely simulator of the intestinal microbial ecosystem (SHIME) (Kemperman et al., 2013).

There are five dietary intervention studies available including a human trial investigating possible effect of dietary polyphenols on the growth of *A. muciniphila* in the gut. The results however, were inconsistent (as shown in Table 1): two showed that oral intake of dietary polyphenols promoted *A. muciniphila* abundance, the other three including the human study showed no effects. Specifically, dietary supplementation of cranberry extract and Concord grape polyphenols increased *A. muciniphila* abundance in feces from 2% to over 30% (OTU sequences) and from 6.2% to 49.1% on the high-fat fed DIO mice, respectively (Anhê et al., 2015; Roopchand et al., 2015). Whereas the intake of pomegranate extract, green tea extract, and whole California table grape showed no effect on *A. muciniphila* abundance of healthy humans or DIO mice (Axling et al., 2012; Baldwin et al., 2016; Li et al., 2015). The inconsistent results suggest that the *A. muciniphila*-promoting effects of dietary polyphenols

are highly depended on their chemical nature and sources. It is unclear whether the significant activity of cranberry extract and Concord grape polyphenols can be translated to humans and more importantly what specific polyphenols are responsible for such activities. It should be noted that although green tea powder showed no effect on *A. muciniphila* abundance, a reduction in body weight gain and other metabolic benefits were observed in high-fat fed obese mice (Axling et al., 2012). Also, in the human study, although pomegranate extract did not change *A. muciniphila* abundance in the feces, the bacterium was 33-fold and 47-fold higher in stool samples of the subjects who are able to produce pomegranate metabolite urolithin as compared to non-producers at baseline and after 4 weeks dietary treatment, respectively. This suggested that *A. muciniphila* might play an important role in the breakdown of phenolic compounds in the intestine and specific group of humans may benefit more from polyphenols for promoting *A. muciniphila* (Li et al., 2015). In summary, since dietary polyphenols are so diverse and abundant, a validated *in vitro* SHIME model would be very helpful as a quick tool to screen and detect which polyphenols are effective in promoting *A. muciniphila* growth, then animal and human studies could be followed up for verifying their activities.

## 7. Metformin consistently increased *A. muciniphila* abundance

Metformin has been used as a first-line drug for treatment of type 2 diabetes (Nauck et al., 2009). Despite its nature as the most popular anti-diabetic drug, the therapeutic mechanisms are not fully understood (Zhou et al., 2001). Interestingly, recent human and animal studies revealed that metformin was able to modulate the gut microbiota and this effect was associated with its anti-inflammatory and anti-obesity as well as its therapeutic efficacy on glucose metabolism (Lee & Ko, 2014; Napolitano et al., 2014; Shin et al., 2014; Zhou et al., 2016). Although no human trails available on *A. muciniphila*, all animal studies consistently showed that metformin significant promoted *A. muciniphila* abundance (as shown in Table 2).

These studies showed that high-fat diet significantly reduced *A. muciniphila* in C57BL/6 J mice. Oral treatment of metformin (100–300 mg/bw) either in drinking water or oral gavage was able to restore its abundance after 4–10 weeks of intervention. Interestingly, metformin treatment had no effect on abundance of control diet fed mice (Shin et al., 2014). The glycemic control and other metabolic disorders associated with diet-induced obesity were significantly improved after metformin treatment. However, it is not known whether the benefits of metformin are mediated through the stimulation of *A. muciniphila.* One of the most significant findings in these studies is that the pretreatment of a combination of antibiotics (carbenicillin, metronidazole, neomycin and vancomycin) on HFD-fed mice before metformin treatment abolished the metformin activity (Shin et al., 2014), which strongly suggesting that the gut bacteria (i.e. *A muciniphila*) play an important role mediating metformin activity. Further, exogenous LPS administration (subcutaneous injection, 50 μg/kg/day for 5 days) nearly completely blocked all these beneficial effects of metformin on glucose metabolism, insulin signaling and redox status in the mice (Zhou et al., 2016), indicating that LPS also plays an essential role in mediating metformin activity. Previous studies also consistently showed that *A. muciniphila* administration significantly reduced serum LPS levels (Everard et al., 2013; Li et al., 2016). There are currently no

reports showing that metformin could directly stimulate mucin production. However, metformin treatment the number of mucin-producing goblet cells increased upon which also increased *A. muciniphila* abundance in mice (Shin et al., 2014). It is likely that metformin promotes *A. muciniphila* resulting in increase of goblet cells because *A. muciniphila* supplementation without metformin also stimulated production of goblet cells in mice (Shin et al., 2014). These findings raise a hypothesis that anti-diabetic activity of metformin is mediated by modulation of gut microbiota, especially the increase of *A muciniphila*, resulting in a reduction in serum LPS levels which in turn reduces inflammation and metabolic disorders. The first evidence that metformin modulated the human gut microbiome profile in diabetes patients was reported by Napolitano et al. (2014), however, *A. muciniphila* was not investigated in this study. Human studies are urgently needed to confirm whether metformin is able to stimulate the abundance of *A. muciniphila* in obese humans and its role in mediating metformin activity.

## 8. Rhubarb extract promoted *A. muciniphila* abundance

Rhubarb (Da Huang) is a well-known Chinese herbal medicine used as a laxative for treatment of constipation, jaundice, gastrointestinal hemorrhage, and ulcers (Huang, Lu, Shen, Chung, & Ong, 2007; Matsuda et al., 2001). Rhubarb mainly contains anthraquinone derivatives which have been reported with anticancer and hepatoprotective activities (Huang et al., 2007; Zhao, Wang, Zhou, Shan, & Xiao, 2009). A recent paper however showed that Rhubarb extract modified gut microbiota of a standard diet (AIN93M)-fed DIO mice (Neyrinck et al., 2016). Supplementation of Rhubarb extract (0.3% in a standard AIN93M diet for 17 days) increased the relative abundance of *A. muciniphila* to 38.9% of fecal total bacteria (measured by pyrosequencing of 16sRNA gene) in DIO mice (12-wk-old) mice (as compared to 9.4% for mice on the standard diet only). The increase was very remarkable considering the treatment only lasted for 17 days. Coincidentally, the relative abundance of *Firmicutes* was reduced to 24.1% (from 48.7% in control mice) (Neyrinck et al., 2016). Rhubarb extract also improved intestinal homeostasis and alcohol-induced oxidative stress and inflammation in the liver (Neyrinck et al., 2016). It remains to be determined that how Rhubarb extract could induce such a drastic change on gut microbiota especially the increase of *A. muciniphila*. It should be pointed out that the fiber content in the rhubarb extract is very low (in case of this study, its concertation in the diet is 0.08%), making it unlikely to be effective (Neyrinck et al., 2016). Similarly, the polyphenols in rhubarb extract may not contribute to the microbiota-modifying effect due to its low concentration. Therefore, it is likely the anthraquinone derivatives in Rhubarb extract are responsible for modulating mice gut microbiota and increasing *A. muciniphila* abundance. Further studies using purified Rhubarb anthraquinones are necessary to confirm whether anthraquinones are promising dietary components for modulating gut microbiota and promoting *A. muciniphila*.

## 9. Caloric restriction (CR): inconsistent results from human and animal studies

Caloric restriction (CR) has been known for increasing longevity in mammals and reducing risk of age-associated diseases including cancer, atherosclerosis, and diabetes (Cohen et al.,

2004; Colman et al., 2009; Mattison et al., 2012), though the exact mechanisms are not known. A recent animal study showed that neonatal CD1 mice in undernutrition condition (timed separation of pups from dams: 12 h of separation per day for 11 days) resulted in a major phylum-level shifts in the distal intestinal microbiota, *A. muciniphila* being increased most significantly (by 50-fold) (Preidis et al., 2015). However, a more recent human study showed an opposite effect that CR actually reduced *A. muciniphila* abundance on obese or overweight subjects. The study on 49 human subjects (11 overweight and 38 obese) found that *A. muciniphila* at baseline was inversely related to fasting glucose, waist-to-hip ratio and subcutaneous adipocyte diameter (Dao et al., 2016). However, CR (CR diet with fibers and protein for 6 weeks) resulted in a decrease in *A. muciniphila* abundance in the Akk HI group (*A. muciniphila* abundance above median) and no change in the Akk LOW group though CR significantly improved insulin sensitivity and other clinical parameters in all groups (Dao et al., 2016). Nevertheless, the authors showed that subjects with higher baseline *A. muciniphila* exhibited more significant improvement in clinical parameters after CR, suggesting an interaction between CR and *A. muciniphila* (Dao et al., 2016). There are only two studies available up-to-date and the information is very limited about the impact of CR on gut microbiota and *A. muciniphila*, and their interactions warrants further investigations.

## 10. Selective antibiotic treatment remarkably promoted *A. muciniphila* abundance in humans and mice

Antibiotic treatment often result in significant change in the bacterial diversity of the gut (Hooper & Gordon, 2001; Manichanh et al., 2010; Pérez-Cobas et al., 2013). Recently, two studies (one on mice and one on humans) showed that antibiotic treatment was able to promote *A. muciniphila* as the most abundant bacterium in the gut (Dubourg et al., 2013; Hansen et al., 2012a, 2012b). Oral treatment of vancomycin on non-obese diabetic (NOD) mice (either from birth until weaning (day 28) or from 8 weeks of age until onset of diabetes) significantly reduced the abundance of once dominated *Firmicutes* and *Bacteroidetes*, promoting *Verrucomicrobia* (all reads within this phylum belonged to *A. muciniphila*) to be the most abundant phylum (>80%) in both groups of vancomycin-treated mice (Hansen et al., 2012a, 2012b). NOD mice spontaneously started to develop insulitis at 3–5 weeks of age (Tisch et al., 1993). Interestingly, vancomycin-treated NOD mice also had lower fasting glucose and cumulative diabetes incidence (73–75%) as compared to untreated NOD mice (93%) (Hansen et al., 2012a, 2012b), suggesting *A. muciniphila* promoted by vancomycin treatment may be involved. In an another study, *Verrucomicrobia* phylotype (*A. muciniphila*) was found unexpectedly in >40% of the total gut microbiota (in feces) from two patients who received a broad-spectrum antibiotic treatment (proportions of 44.9% for patient A with *Coxiella burnetii* vascular infection, receiving combination of doxycycline, hydroxychloroquine, piperacillin/tazobactam and teicoplanin and 84.6% for patients B admitted to the Intensive Care Unit, receiving10-day course of imipenem) (Dubourg et al., 2013). Interestingly, neither patient presented significant gastrointestinal disorders despite such a significant change in gut microbiota (Dubourg et al., 2013). An *in vitro* antibiotic susceptibility test showed that *A. muciniphila* was susceptible to imipenem, piperacillin/tazobactam, and doxycycline but was resistant to vancomycin, metronidazole, and penicillin

G (Dubourg et al., 2013). It is surprising that *A. muciniphila* is susceptible to imipenem but *A. muciniphila* represent over 80% of total microbiota in the stool sample of patient B who received 10-day course of imipenem. Nevertheless, both the studies demonstrated that antibiotic treatment can drastically change gut microbiota, especially *A. muciniphila*.

## 11. High fat diet and alcohol could reduce abundance of *A. muciniphila*

Previous studies have consistently shown that high-fat diet significantly reduced *A. muciniphila* abundance in different animal models (Anhê et al., 2015; Axling et al., 2012; Everard et al., 2013; Li et al., 2016; Roopchand et al., 2015; Zhou et al., 2016). A treatment of high-fat diet (60% fat) for as short as 8 weeks on DIO mice led to a 100-fold decrease of *A. muciniphila* (Everard et al., 2013). Alcohol intake could also negatively affect *A. muciniphila* (Neyrinck et al., 2016). Acute alcohol administration (30% w/v, 6 g/kg body weight) caused 100-fold decrease in the relative abundance of *A. muciniphila* in fecal bacterial content of DIO mice (reduced from 9.3% (absolute abundance: about 9.8 $Log_{10}$ cell number/g feces) to 3.8% (absolute abundance: about 7.8 $Log_{10}$ cell number/g feces), accompanied by increased inflammation and oxidative stress (Neyrinck et al., 2016). Interestingly, supplementation of ground dietary flaxseed (10% in an AIN-93G basal diet for 3 weeks) caused a 30-fold reduction in *A. muciniphila* abundance in fecal total bacteria of DIO male mice (Power et al., 2016), despite that flaxseed supplementation improved intestinal barrier integrity by promoting colon goblet cell density, mucus production, and cecal short chain fatty acid levels (Power et al., 2016).

## 12. Summary

We have carefully examined a total of 24 the up-to-date available dietary intervention studies in search for evidence and strategies to increase *A. muciniphila*, a beneficial member of gut microbiota in the gut. Available evidence from animal studies showed that viable *A. muciniphila* or prebiotics (FOS) was able to consistently promote *A. muciniphila* abundance in the gut, suggesting a great potential for future development of dietary intervention approaches using viable bacterium or FOS for increasing gut *A. muciniphila.* Supplementation of *B. animalis* could also increase *A. muciniphila* by producing SCFA and facilitating mucin growth to feed the bacterium. Metformin and antibiotics treatment (vancomycin) also significantly promote *A. muciniphila* abundance in the gut but these strategies are not suitable for general public. Rhubarb extract is promising but more research is needed to confirm its activity and another concern about Rhubarb is that it is not a typical dietary ingredient. Dietary polyphenols are inconsistent, cranberry extract and Concord grape polyphenols are active but green tea and whole grape showed no effect. The inconsistency may be related to their difference in polyphenol profile but to identify the active polyphenols is challenging due to their abundance and diversity in the extract. It should also be noted that to maintain *A. muciniphila* abundance in the gut one may want to avoid high-fat diet and heavy alcohol consumption, though the results were based on the measurement of relative abundance of gut microbials.

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**Table 1**

Effects of dietary polyphenols on *A. muciniphila* abundance.

| Dietary polyphenols | Subjects/Animal models | Treatment | Effects on *A. muciniphila* | Other effects | References |
|---|---|---|---|---|---|
| Pomegranate extract | Healthy humans | 1 g/d for 4 weeks | No effect | N/A | Li et al. (2015) |
| Cranberry extract | DIO mice | 200 mg/kg bw with a high-fat/high sucrose diet for 8 weeks | Increase relative abundance (operational taxonomic unit sequences) from 2% to over 30% in feces | Prevent increase of LPS and intestinal inflammation, body weight gain, visceral obesity, insulin resistance induced by high-fat/high sucrose diet | Anhê et al. (2015) |
| Green tea powder | DIO mice | 4% in a high-fat diet for 22 weeks | No effect | Reduced the body fat content and hepatic triacylglycerol and cholesterol accumulation | Axling et al. (2012) |
| Concord grape polyphenols | DIO mice | 1% in a high-fat diet for 13 weeks | Increased the relative abundance of *A. muciniphila* in cecal sample: from 6.2 to 49.1% 16 S rRNA sequences; fecal sample: from 7.5 to 54.8% 16 S rRNA sequences | Reduced serum LPS by 81%, inflammation, body weight gain, adiposity, glucose intolerance, increased gut barrier function | Roopchand et al. (2015) |
| Whole grape | DIO mice | 3–5% California table grape in high-fat diet for 11 weeks | No significant effect | Reduced adiposity and markers of hepatic lipogenesis and alters gut microbiota in butter fat-fed mice | Baldwin et al. (2016) |

**Table 2**

Effects of metformin on *A. muciniphila* abundance.

| Metformin | Subjects/Animal models | Treatment | Effects on *A. muciniphila* | Other effects | References |
|---|---|---|---|---|---|
| Oral gavage | DIO mice | 300 mg/kg bw with a high-fat diet for 6 weeks | Increase relative abundance from 0.3% to 2.9% in feces (versus 2.3% in control diet fed mice) | Improved glycemic control, Increased the number of mucin-producing goblet cells, reduced serum LPS; however, pretreatment of antibiotics abolished metformin activity | Shin et al. (2014) |
| In drinking water | DIO mice | 100 mg/kg/d with a high-fat diet for 4 weeks | Restored the reduced abundance in the feces of (no specific data on number/ percentage) | Improved insulin sensitivity & glucose control, reduced serum LPS; exogenous LPS administration abolished all metformin activity | Zhou et al. (2016) |
| Oral gavage | DIO mice | 300 mg/kg bw with a high-fat diet for 10 weeks | Increase relative abundance from 0.7% to 12.4% in feces | Improved metabolic disorders, increased the relative abundance of the phylum *Bacteroidetes* | Lee and Ko (2014) |

*Review Article*

# Manipulating Gut Microbiota Composition to Enhance the Therapeutic Effect of Cancer Immunotherapy

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## Abstract

In the past decade, a growing set of immunotherapies including immune checkpoint blockade, chimeric antigen receptor T cells, and bispecific antibodies propelled the advancement of oncology therapeutics. Accumulating evidence demonstrates that immunotherapy could eliminate tumors better than traditional chemotherapy or radiotherapy with lower risk of adverse events in numerous cancer types. Unfortunately, a substantial proportion of patients eventually acquire resistance to immunotherapy. By analyzing the differences between immunotherapy-sensitive and immunotherapy-resistant populations, it was noticed that the composition of gut microbiota is closely related to treatment effect. Moreover, in xenograft models, interventional regulation of gut microbiota could effectively enhance efficacy and relieve resistance during immunotherapy. Thus, we believe that gut microbiota composition might be helpful to explain the heterogeneity of treatment effect, and manipulating gut microbiota could be a promising adjuvant treatment for cancer immunotherapy. In this mini review, we focus on the latest understanding of the cross-talk between gut microbiota and host immunity. Moreover, we highlight the role of gut microbiota in cancer immunotherapy including immune checkpoint inhibitor and adoptive cell transfer.

## Keywords

gut microbiota, immunotherapy, PD-1, PD-L1, CTLA-4, adoptive cell transfer

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## Introduction

The gut microbiota contains a large number of microorganisms populating in gastrointestinal tract such as bacteria, fungi, protozoa, virus, phages, and archaea.[1,2] It is generally believed that the gut flora consists of essential and opportunistic bacteria.[3] The essential bacteria are beneficial to humans and participate in fermenting undigested carbohydrates and endogenous mucus, synthesizing short-chain fatty acids (SCFA) and vitamins, and defending against infection by pathogens.[4-6] On the contrary, overgrowth of opportunistic bacteria could lead to infection.[3] The imbalance between essential and opportunistic bacteria results in gut dysbiosis, which usually refers to the compositional and functional alteration in microbiota driven by environmental or host-associated factors.[7] It has been well established that gut dysbiosis relates to some diseases including inflammatory bowel disease, nonalcoholic fatty liver disease, neurodegenerative disorders, and metabolic disease.[8-13] Besides, gut dysbiosis is regarded as an important risk factor promoting tumor initiation and development.[7] Some specific bacteria have been confirmed as carcinogens such as *Helicobacter pylori* for gastric cancer and *Salmonella typhi* for biliary cancer.[14,15] The carcinogenic role of *H pylori* is related to its genotoxic effect, which further results in chronic inflammation and hyperactive proliferation signaling pathways in mucosal cells.[14] Following long-term stimulation, *H pylori* could induce malignant transformation in gastric epithelia and mucosa-associated lymphoid tissues.[14] Moreover, it has

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been verified that gut microbiota closely associate with host immunity.[16,17] On the one hand, the gut microbiota participates in the development of the host immune system.[18] On the other hand, the composition of gut microbiota is modulated by host immunity.[19]

Accumulating evidence demonstrates that gut microbiota could affect the therapeutic effect of multiple cancer treatments including chemotherapy, radiotherapy, as well as immunotherapy.[20-22] The results of in vitro and in vivo studies showed that gut microbiota could regulate the efficacy of chemotherapy by multiple approaches, including (1) Translocation: bacteria cross chemotherapy-induced damaged gut mucosal barrier and enter peripheral lymph nodes; (2) Immunomodulation: gut microbiota promotes chemotherapy-related inflammation; (3) Metabolism and enzymatic degradation: gut microbiota could directly or indirectly modify the structure of pharmaceuticals, which might enhance or abrogate the efficacy of treatment and introduce toxic compounds; (4) Reduced diversity: chemotherapy tends to reduce to the diversity of gut microbiota and leads to the formation of pathogen-dominant gut flora and higher risk of gastrointestinal reactions.[20] However, the exact mechanism by which gut microbiota modulates the efficacy of immunotherapy is still unclear.

Benefiting from the development of sequencing technology, it is now possible to analyze the composition of the microbiota.[23] Commonly, 16S rRNA and metagenomic shotgun sequencing are adopted for taxonomic assignment.[24] Taxonomic identification by 16S rRNA is based on the comparison between detection results and known database. Therefore, with 16S rRNA sequencing, it is difficult to identify unknown species.[24] Compared with 16S rRNA sequencing, metagenomic shotgun sequencing could directly analyze the whole genomic context, which could be used for taxonomic identification and function analysis.[24] Moreover, more and more microbiome studies utilize long-read sequencing that could overcome the limitations of next-generation sequencing such as identifying structural variants, repetitive regions, alleles, and highly homologous genomic regions. Given the vital role of gut microbiota in anticancer therapy, identifying efficacy-related bacteria provide a novel perspective to counteract drug resistance especially for immunotherapy.

## The Cross-Talk Between Gut Microbiota and the Host Immune System

The cross-talk between gut microbiota and immunity is complicated. Host immunity not only sustains tolerance to symbiotic commensals and food antigens but also recognizes opportunistic bacteria and defends against pathogen infection.[25] In the meantime, the influence of gut microbiota on the host immune system is multifaceted, from localized immune response to systemic innate or adaptive immunity.[25] It was observed that mice that were bred and raised in a sterile environment (germ-free mice) were prone to harbor deficiencies in the development of gut-associated lymphoid tissues especially Peyer's patches (PP) and isolated lymphoid follicles.[26] Besides, depleting gut microbiota by broad-spectrum antibiotics inhibited murine bone marrow hematopoiesis and decreased the abundance of hematopoietic stem cells or multipotent progenitors.[27]

### *Gut Mucosal Immune System*

The gut mucosal immune system contains organized lymphoid tissues located in the gut mucosal epithelium, lamina propria, and mesentery including PP, isolated lymphoid follicles, and mesenteric lymph node.[28-30] Among them, the mucus layer and mucosal epithelium comprise the physical barrier of gut mucosal immunity.[31] It is generally believed that the mucus is mainly produced by goblet cells.[32] During mucus secretion, goblet cells in the small intestine can sense and sample luminal content.[32] In a manner that has not been well studied, actively secreting goblet cells take up antigenic materials and deliver them to dendritic cells (DCs) in lamina propria.[32] Notably, the mucus contains abundant antimicrobial peptides that effectively clear bacterial clones on gut epithelium. As a part of intestinal innate immunity, Paneth cells in the base of intestine crypts are main producers of antimicrobial peptides.[33] Decreased antimicrobial peptides lead to elevated bacterial colonization and hyperactive adaptive immune response.[34] Mucosal epithelial cells under the mucus layer not only directly isolate gut microbiota but also secrete cytokines and chemokines to regulate the mucosal immune system.[31] In mucosal epithelium, innate lymphoid cells (ILCs) play an important role in regulating the magnitude of inflammation and maintaining intestinal homeostasis.[31] By secreting interleukin (IL)-22, ILCs promote healing during infection and counteract the damaging effect of immune response.[35] In the meantime, ILCs also stimulate the production of antimicrobial peptides to kill gram-positive bacteria.[36]

Peyer's patches are the core component of gut-associated lymphoid tissue and are distributed throughout the small intestine.[37] Distinguished from peripheral lymph organs, PP harbor some specialized structures.[37] Notably, there are no afferent lymphatics in PP. Instead, PP are overlain by specialized microfold epithelial cells (termed M cells), which constantly sample and deliver antigens from the lumen into PP.[38] A host of DCs are enriched in the area underneath M cells (subepithelial dome region), capturing and presenting antigens from M cells.[38] Apart from antigen presentation, DCs in PP express retinol dehydrogenase that promotes the production of retinoic acid.[28] Retinoic acid induces the homing of activated T or B cells to intestinal lamina propria by upregulating gut-imprinting molecules

such as CCR9 and integrin α4β7 on lymphocytes.[39-41] Apart from DCs in PP or isolated lymphoid follicles, it has been detected that a subset of DCs populate the gut mucosal epithelium, which are called "intraepithelial DCs."[42] Intraepithelial DCs are characterized by CX3CR1 expression and directly capture antigens from the intestinal lumen by their transepithelial dendrites.[43] After activation, DCs traffic to mesenteric lymph nodes and induce the polarization of naïve $CD4^+$ T cells toward inducible regulatory T cells (iTreg) or Th1/Th17 cells.[25] After education (a process also known as imprinting, referring to how naïve T cells learn to express homing receptors for skin, gut, or other tissues) in mesenteric lymph node, most newly generated iTreg, Th17, and Th1 cells home to the gut by the guidance of gut-imprinting molecules, while a part of lymphocytes circulate systemically.[25,44]

### *The Regulatory Effect of Gut Microbiota on the Gut Mucosal Immune System*

The gut microbiota and its metabolites have a broad and profound influence on multiple aspects of the host gut mucosal immune system.[1] It has been reported that human commensal *Bacteroides fragilis* could induce the differentiation of $CD4^+$ naïve T cell into Treg and enhance the secretion of anti-inflammatory cytokines (eg, IL-10).[45] *B fragilis*–induced intestinal immune tolerance is dependent on polysaccharide A and toll-like receptor 2 signaling, favoring to maintain gut homeostasis.[45] Similarly, Cebula et al found that most colonic Treg cells belonged to thymus-derived Tregs, which recognized the antigenic materials from bacteria such as *Clostridiales, Bacteroides*, and *Lactobacillus*.[46] Simultaneously, the colonic Treg cells could maintain tolerance to these bacteria.[46] It was notable that antibiotic-mediated alteration in gut microbiota composition (mainly reducing the members of *Clostridiales*) significantly decreased the abundance of colonic Treg cells and changed the T cell receptor (TCR) repertoire of these thymus-derived Tregs.[46] Contrary to *B fragilis*, some commensals modify gut immunity toward a pro-inflammatory direction, such as commensal segmented filamentous bacteria and adherent invasive *Escherichia coli*.[47-49] Segmented filamentous bacteria promote the development of Th17 and induce the production of IL-17 in $ROR\gamma t^+$ $CD4^+$ T cells.[50]

Bacterial metabolites have been documented as a vital regulator of gut immune response as well. SCFAs including acetate, propionate, butyrate, and isobutyrate are end products of the fermentation activity of intestinal microorganisms.[51,52] A growing body of studies demonstrated that SCFAs enhanced the generation and immune inhibitory capability by counteracting the effect of histone deacetylase and promoting acetylation of Foxp3 locus.[53] In addition, butyrate-mediated inhibition of histone deacetylase could interfere with some lipopolysaccharides-responsive signaling pathways in DC, further enhancing the conversion from naïve $CD4^+$ T toward a Treg population.[1]

### *The Influence of Gut Microbiota on Host Systemic Immunity*

The binding between pathogen-associated molecular patterns and pattern recognition receptors (eg, toll-like receptors) together with bacteria-derived metabolites (eg, SCFAs) influence the local immune response in gut.[25] However, the regulatory effect of gut microbiota is not just limited to the localized mucosal immune system. Actually, gut microbiota have a substantial effect on host systemic immunity via cytokine secretion, cross-reactivation, lymphocyte homing, and recirculation.[25] By consistent antigen sampling of interdigitation of DCs and M cells, the stimulation of pathogen-associated molecular patterns propel the maturation and activation of DCs.[25] There are abundant draining lymph nodes in the mesentery of the small intestine and colon where the differentiation of naïve $CD4^+$ T cells can be modulated by DCs.[25] Apart from inducing $CD4^+$ T cell differentiation (especially toward Tregs and Th17 cells), DCs might stimulate $CD8^+$ T cells in mesenteric lymph nodes.[25] Moreover, a subset of activated DCs in the gut enter into circulation and induce a broader immune response.[25] Besides, as mentioned above, some primed lymphocytes in mesenteric lymph nodes could subsequently enter the circulation as well. Due to cross-reactivity, gut microbiota–specific lymphocytes recognize and attack distant tissues with similar antigenic epitopes.[54,55] Moreover, cytokines afforded by gut mucosal immune response might be secreted into circulation and set immunological tone, promoting host immunity to robustly respond to pathogens and to sustain the tolerance to innocuous commensals.[56]

## Anticancer Immune Response and Immunotherapy

During malignant transformation, accumulating mutations increase the immunogenicity of tumor cell by generating tumor-associated antigen or neoantigen.[57-59] In the condition of intact immune surveillance, host immunity could recognize and clear these immunogenic materials.[60] However, a proportion of cancer cells could escape from immune elimination via various manners such as losing immunogenic antigens, dysregulating antigen presentation machinery, activating immune checkpoint signaling pathway, recruiting pro-tumor immune cells, and transforming growth factor β signaling–mediated exclusion of $CD8^+$ T cells by the tumor parenchyma.[61-63] As a result, antitumor immune response is impaired and tumor cells proliferate uncontrollably.[64] Immunotherapy is aimed at restoring robust immune surveillance through regulating the balance between immunosupportive factors and immunosuppressive factors.[65] The

efficacy of immunotherapy could be affected by various determinants such as antigen presentation, T cell priming and activation, T cell trafficking and infiltration, as well as cytotoxicity activity of tumor infiltrating lymphocytes (TILs).[60,66-68] Therefore, interventions affecting any processes of the cancer-immunity cycle could influence the efficacy of immunotherapy.

### *Immune Checkpoint Inhibitor*

The activation of tumor-specific T cells needs 2 steps. First, TCR selectively binds to major histocompatibility complex I with anchored antigen peptides.[69] Then, synergizing with co-stimulatory signals such as CD28, ICOS, and OX40, the activation signal of TCR/CD3 complex is further amplified and ultimately leads to the priming and activation of T cell.[69] Contrarily, co-inhibitory signals (also known as immune checkpoints) including programmed cell death-1 (PD-1), cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), T cell immunoglobulin domain and mucin domain-3 (TIM-3), and lymphocyte activation gene-3 (LAG-3) undermine T cell activation by intracellular immunoreceptor tyrosine–based inhibition motif (ITIM) to counteract TCR/CD3- or CD28-mediated tyrosine phosphorylation (Figure 1).[70-72] Cancer cells tend to upregulate the activity of co-inhibitory signaling pathways to escape immune surveillance.[73,74] Immune checkpoint inhibitors (ICIs) alleviate immune tolerance to tumor antigens and reinvigorate the antitumor response. Anti-PD-1/PD-L1 and anti-CTLA-4 have been successfully applied in multiple cancers.[75-80] Nevertheless, there is a great potential to enhance the anticancer effect of ICI.

### *Adoptive Cell Transfer*

The therapeutic effect of ICI is highly dependent on preexisting tumor-specific immune cells.[66] However, for some poorly immunogenic cancers, it is hard to eradicate cancer cells via ICI. In the context of an immune ignorant microenvironment, the injection of tumor-specific immune cells might be a reasonable strategy.[81] Generally, adoptive cell transfer (ACT) could be deployed by 2 approaches: (1) expanding TILs in vitro, then reinfusing obtained TILs into patients; (2) isolating T cells from patients' peripheral blood, genetically modifying T cells to express chimeric antigen receptor or specific TCR.[82-85] ACT especially CAR-T exhibits potent anticancer effect in multiple hematological malignancies.[86-88] However, the efficacy of ACT is limited to solid tumors, which is mainly attributed to unfavorable cytokine milieu, dysregulated Treg/T effector cell ratio, limited immune cell trafficking, as well as antigen heterogeneity.[89] Interventions modulating the immune microenvironment and expanding T cell clones would be helpful to overcome the obstacles to ACT application in solid tumors.[83,90]

## Gut Microbiota Modulates the Efficacy of Immunotherapy

The gut microbiota possesses a broad range of regulatory effects on multiple immune effectors including the maturation of DCs, the differentiation of T cells, as well as the secretion of cytokines, which might regulate anticancer immunity (Figure 2).[25,91] A series of studies indicated that gut microbiota composition is closely associated with the efficacy of cancer immunotherapy (summarized in Table 1).[91]

### *The Role of Gut Microbiota in Anti-PD-1/PD-L1 Treatment*

Anti-PD-1/PD-L1 treatment blocks the negative signal transduced by intracellular domains of PD-1 (ITIM and ITSM).[92] PD-1/PD-L1 blockade not only promotes TCR/CD3- or CD28-mediated T cell activation but also enhances T cell survival and proliferation via upregulating Ras-Raf-MAPK and PI3K-AKT signaling pathways.[93,94] Anti-PD-1/PD-L1 therapy has been approved for multiple types of cancers such as melanoma, non–small cell lung cancer, and kidney cell cancer.[95-98] It has been verified that biomarkers including PD-L1 expression level, TIL status, and mismatch repair system deficiency highly correlate with the treatment effect of anti-PD-1/PD-L1.[66] Besides these factors mentioned above, the gut microbiota contributes to the heterogeneity of therapeutic reaction as well.[91]

As early as 2015, Sivan et al noticed that the abundance of some special commensal bacteria was related to anti-PD-1 treatment effect in a mouse model.[99] Researchers compared the efficacy of anti-PD-1 treatment in genetically similar mice (C57BL/6) from 2 different facilities (JAX and TAC) that harbored significantly different gut microbiota.[99] The results showed that tumors grew more slowly and were more sensitive to anti-PD-1 therapy in JAX populations. This difference was attributed to enhanced antitumor immunity in JAX that could be transferred to TAC mice by cohousing or transplanting JAX fecal suspension to TAC.[99] Based on the 16S rRNA sequencing technique, it was detected that markedly increased abundance of *Bifidobacterium* in JAX primarily led to elevated levels of TIL and better treatment response to anti-PD-1 therapy.[99] Administration of commercially available *Bifidobacterium* including *Bifidobacterium breve and Bifidobacterium longum* significantly promoted tumor control especially combined with anti-PD-1 treatment.[99] To interrogate the mechanism by which *Bifidobacterium* administration synergized with anti-PD-1 treatment, researchers monitored the abundance and function of tumor antigen–specific $CD8^+$ T cell.[99] It was observed that *Bifidobacterium*

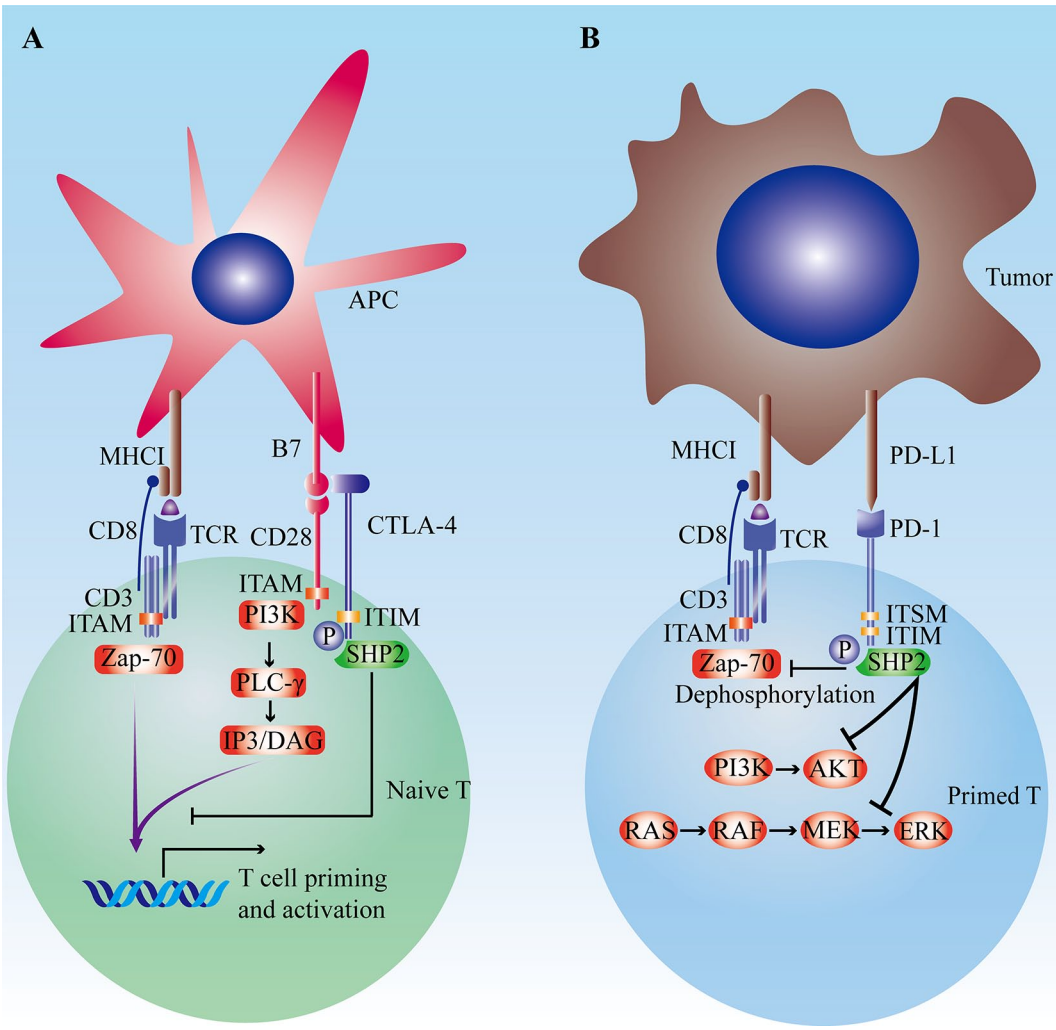


**Figure 1.** The regulatory function of immune checkpoints. (A) The role of CTLA-4 in the priming and activation of naïve T cells. The activation of T cells is driven by stimulatory signals of TCR/CD3 complex and CD28. CTLA-4 could competitively antagonize co-stimulatory signal of CD28-B7 pathway and subsequently inhibits the T cells activation. (B) PD-1/PD-L1 signaling pathway. PD-1/PD-L1 signaling pathway to counteract CD3- or CD28-mediated tyrosine phosphorylation by ITIM and ITSM. Besides, PD-1 could disturb T cell proliferation and survival by inhibiting PI3K-AKT and Ras-Raf-MEK-ERK pathway.

Abbreviations: APC, antigen presentation cell; CTLA-4, cytotoxic T-lymphocyte–associated protein 4; ITIM, intracellular immunoreceptor tyrosine–based inhibition motif; ITSM, immunoreceptor tyrosine–based switch motif; MHC, major histocompatibility complex; PD-1, programmed cell death-1; TCR, T cell receptor.

remarkably upregulated the level of tumor-specific $CD8^+$ T cell and interferon (IFN)-γ secretion.[99] In addition, in an in vitro experiment, DCs obtained from TAC receiving *Bifidobacterium* treatment showed improved capability to induce T cell priming and activation.[99]

Motivated by the encouraging finding in mouse models, a series of studies were deployed to explore the relationship between gut microbiota and anti-PD-1 treatment in cancer patients. Gopalakrishnan et al analyzed gut microbiota of melanoma patients undergoing anti-PD-1 treatment.[100] The results demonstrated that gut microbial diversity was higher in responders, and the α-diversity (parameter reflecting bacterial community richness and evenness) of fecal samples was positively correlated to progression-free survival (PFS) time.[100] Further analysis indicated that the level of *Faecalibacterium* (belonging to the *Ruminococcaceae* family, *Clostridiales* order) was higher in responders while *Anaerotruncus colihominis, Bacteroides thetaiotaomicron* (belonging to *Bacteroidales* order), and *Escherichia coli* were significantly enriched in nonresponders.[100] In addition, it was found that the abundance of *Faecalibacterium* is positively correlated with the level of $CD8^+$ TIL ($R^2 = 0.42$,

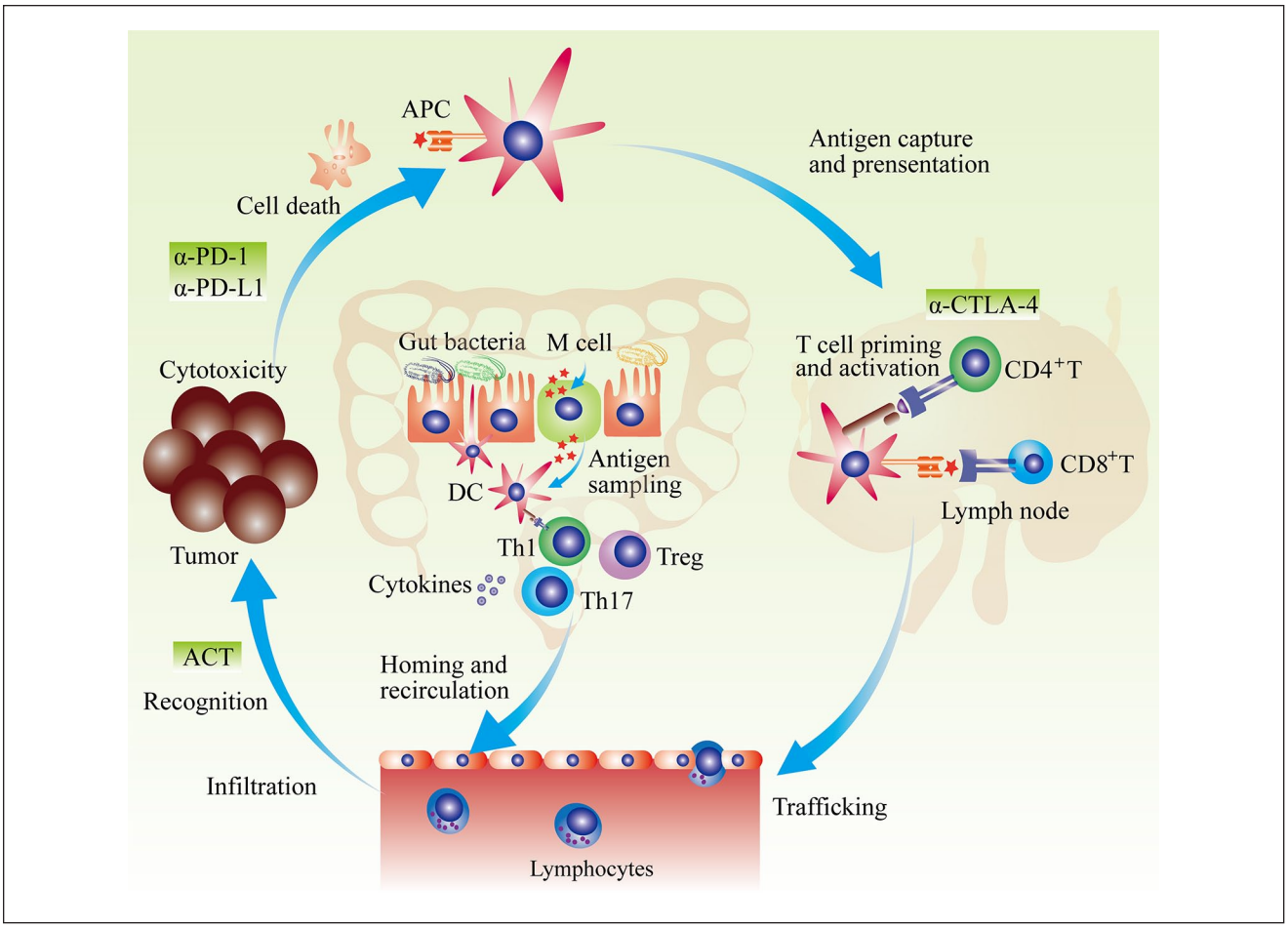


**Figure 2.** Gut microbiota and anticancer immunotherapy. APCs capture and recognize dead tumor cell–derived antigens. Then, in peripheral lymphatic organs, APCs present possessed antigens and activate naïve T cells. Primed T cells migrate and infiltrate into tumor. After recognition of tumor antigen, activated T cells kill tumor cells. Factors interfering any producer of anticancer-immunity cycle could result in cancer immune escape. Generally, anti-PD-1/PD-L1 treatment mainly enhance tumor-killing activity; anti-CTLA-4 primarily promotes the priming and activation of T cells; and adoptive cell transfer mainly induces T cell clones recognizing tumor cells. Gut microbiota could affect anticancer immunotherapy by multiple manners. Gut microbiota–derived antigens could regulate the development and function of DC in gut, which further influences gut mucosa immunity. Induced immune response such as Th1-skweing immunity, Th17 polarization, Treg differentiation, and cytokines secretion could enter into circulation and influence the effect of systemic anticancer immunotherapy.

Abbreviations: ACT, adoptive cell transfer; APC, antigen presentation cell; α-CTLA-4, anti-cytotoxic T-lymphocyte–associated protein 4; α-PD-1, anti-programmed cell death-1; DC, dendritic cell; Treg, regulatory T cell.

$P < .01$).[100] The immune cell detection in circulation showed that increased gut *Faecalibacterium* accompanied elevated $CD4^+$ or $CD8^+$ T cells.[100] Conversely, the level of systemic *Bacteroidales* positively related to the quantity of myeloid-derived suppressor cells and Tregs.[100] Confirmed by fecal transplantation experiments in the mouse model, therapeutic benefit afforded by favorable bacteria was attributed to promoting the formation of hot tumor (according to the status of TILs, tumors can be classified as hot/T cell inflamed or cold/T cell non-inflamed tumors) via increasing local effector immune cells and decreasing suppressive immune cells.[100]

Similarly, Matson et al noticed the influence of gut microbiota on the efficacy of anti-PD-1 treatment in metastatic melanoma patients.[101] Based on an integrative identification method (including 16S rRNA sequencing, metagenomics shotgun sequencing, and species-specific quantitative polymerase chain reaction), researchers observed that some bacteria such as *Bifidobacterium adolescentis, B longum, Collinsella aerofaciens, Enterococcus faecium, Klebsiella pneumoniae, Lactobacillus* species, *Parabacteroides merdae*, and *Veillonella parvula* were significantly enriched in responders, while *Ruminococcus obeum* and *Roseburia intestinalis* were remarkably abundant in nonresponders.[101]

**Table1.** Regulatory Effect of Gut Microbiota on Cancer Immunotherapy.

| Bacterium | Regulatory Effect on Immunity | Influence on Immunotherapy | Author |
|---|---|---|---|
| *Bifidobacterium* | Enhancing the function of DC<br>Upregulating tumor-specific $CD8^+$ T<br>Increasing pro-inflammatory cytokine | Enhancing PD-1 blockade effect | Sivan et al[99] |
| *Faecalibacterium* | Increasing $CD4^+$ and $CD8^+$ T in circulation and in tumor | Enhancing PD-1 blockade effect | Gopalakrishnan et al[100] |
| *Bacteroidales* | Upregulating systemic MDSC and Treg | Impeding PD-1 blockade effect | Gopalakrishnan et al[100] |
| A group of bacteria including *Bifidobacterium adolescentis, Bifidobacterium longum*, and so on. | Elevating the secretion of IFN-γ<br>Increasing $CD8^+$ tumor infiltrating T | Enhancing PD-1 blockade effect | Matson et al[101] |
| *Ruminococcus obeum* and *Roseburia intestinalis* | Enriched in patients resistant to anti-PD-1 treatment | Impeding PD-1 blockade effect | Matson et al[101] |
| *Akkermansia muciniphila* | Increasing $CXCR3^+CCR9^+CD4^+$ T cell<br>Enhancing ability of DC and production of IL12 | Enhancing PD-1 blockade effect | Routy et al[102] |
| *Bacteroides fragilis* | Inducing Th1 immune response and DC maturation | Enhancing CTLA-4 blockade | Vétizou et al[103] |
| *Faecalibacterium* | Promoting development of Treg<br>Upregulating ICOS expression of T cells; | Enhancing CTLA-4 blockade | Chaput et al[104] |
| *Bacteroides* | Leading to baseline systemic inflammation | Impeding CTLA-4 blockade effect | Chaput et al[104] |
| Some species of *Bacteroidetes* | Decreasing DC and IL-12<br>Inducing the formation of cold tumor | Impeding ACT effect | Uribe-Herranz et al[105] |

Abbreviations: ACT, adoptive cell transfer; CTLA-4, cytotoxic T-lymphocyte–associated protein 4; DC, dendritic cell; ICOS, inducible T cell co-stimulator; MDSC, myeloid-derived suppressor cell; PD-1, programmed cell death-1; Treg, regulatory T cell.

Moreover, germ-free mice gavaged with fecal materials from responders had a markedly increased level of $CD8^+$ TIL and secretion of IFN-γ, promoting the formation of an immunosupportive microenvironment.[101]

Around the same time, Routy et al reported the role of gut microbiota in anti-PD-1 treatment resistance.[102] Researchers found that for cancer patients receiving anti-PD-1 treatment, additional oral antibiotic treatment (within 2 months before or 1 month after the start of anti-PD-1 treatment) significantly shortened overall survival (OS) and PFS time.[102] To investigate the relationship between antibiotic-induced dysbiosis and impaired therapeutic effect, researchers compared the gut microbiota composition between responders and nonresponders.[102] Among all bacteria overrepresented in responders, *Akkermansia muciniphila* was most significantly related to patients' response rate ($P = .007$).[102] Besides, immune reactivity of Tc1 or Th1 against *A muciniphila* correlated with improved survival data ($P = .032$).[102] By fecal transplantation and antibiotics treatment in mice, researchers interrogated the influence of gut microbiota and oral antibiotic-induced dysbiosis on anti-PD-1 treatment effect.[102] It was observed that mice receiving fecal transplantation from responders reacted better to PD-1 blockade with increased $CXCL3^+CD4^+$ TILs, while mice receiving fecal transplantation from nonresponders, in germ-free status, or undergoing antibiotic treatment were resistant to PD-1 blockade.[102] Notably, the PD-1 resistance in germ-free or antibiotics-treated mice could be rescued by recolonization of *A muciniphila* and *Enterococcus hirae*.[102] Further exploration showed that accumulated $CXCR3^+CCR9^+CD4^+$ T cells and DC-IL-12 axis–mediated Th1-skewing priming might contribute to the enhanced therapeutic response to PD-1 blockade.[102]

## *The Effect of Gut Commensals on Anti-CTLA-4 Treatment*

CTLA-4 blockade treatment mainly restores the activity of co-stimulatory signaling pathway (CD28-CD80/86) hijacked by CTLA-4. Exploring factors modulating anti-CTLA-4 treatment effect is helpful to enhance treatment response and relieve drug resistance. Vétizou et al found that some special gut bacteria supplements could enhance the effect of CTLA-4 blockade.[103] Researchers noticed that broad-spectrum antibiotic treatment abrogated the antitumor effect of CTLA-4 blockade. Additionally, anti-CTLA-4 antibody could not effectively inhibit tumor progression in germ-free mice indicating that gut microbiota might participate in anti-CTLA-4 treatment.[103] Recolonization of *Bacteroides thetaiotaomicron, B fragilis*, or *Burkholderia cepacia* in germ-free or antibiotic-treated mice rescued CTLA-4 blockade resistance.[103] Further detection showed

that oral gavage of *B fragilis* induced Th1 immune response and DC maturation in tumor-draining lymph node.[103] Besides, adoptive *B fragilis*–specific Th1 cell transfer could partially restore sensitivity to CTLA-4 blockade in germ-free or antibiotic-treated mice.[103] Apart from the enhanced CTLA-4 blockade effect, the recolonization of *B fragilis* and *Burkholderia cepacia* could alleviate treatment-induced colitis.[103]

Later in 2017, Chaput et al verified the regulatory effect of gut microbiota on CTLA-4 blockade in metastatic melanoma patients.[104] In the recruited patients, baseline microbiota composition could herald prognostic status after undergoing CTLA-4 blockade treatment.[104] Overrepresented *Bacteroides* at baseline predicted poor outcomes ($P = .034$), while increased *Faecalibacterium* at baseline indicated long-term benefits ($P = .0092$).[104] Besides, all patients with survival time longer than 18 months could be screened out by gut microbiota composition harboring overrepresented *Ruminococcus* and *Lachnospiraceae* genus (belonging to the *Firmicutes* phylum).[104] Contrary to the observations mentioned above, baseline antibiotic treatment could not disturb the composition of gut microbiota.[104] Then, clustering analysis indicated that patients with gut microbiota containing *Faecalibacterium* or other bacteria belonging to the *Firmicutes* phylum (eg, unclassified *Ruminococcaceae, Clostridium XIVa*, and *Blautia*) tended to possess better clinical outcomes (PFS: $P = .039$; OS: $P = .051$).[104] In line with enhanced CTLA-4 blockade effect, patients with overrepresented *Faecalibacterium* or other *Firmicutes* had increased risk of treatment-induced colitis, especially compared with patients with *Bacteroides*-dominant gut microbiota.[104] To interrogate the mechanisms by which *Faecalibacterium*-dominant gut microbiota composition increased CTLA-4 blockade effect and corresponding adverse events, researchers monitored immune status–related parameters.[104] It was found that patients with *Faecalibacterium*-dominant gut microbiota had lower $CD4^+/CD8^+$ T cells and systemic proinflammatory cytokine levels at baseline, as well as higher ICOS expression on $CD4^+$ T after the start of anti-CTLA-4 treatment.[104] Presumably, *Faecalibacterium* and other *Firmicutes* contributed to decreased systemic inflammation by inducing the development of Treg at baseline.[104] However, as the primary target of anti-CTLA-4 treatment, increased Treg level endowed patients with elevated sensitivity to CTLA-4 blockade as well as decreased risk of treatment-induced colitis.[104]

## Manipulating Gut Microbiota to Enhance Effect of ACT

The therapeutic effect of ACT is limited by peripheral tolerance and immune escape in the tumor microenvironment.[105] Uribe-Herranz et al found that manipulating the composition of gut microbiota could modulate the effect of ACT.[105] Researchers found that the treatment effect of ACT was different in genetically similar mice (C57BL/6) from 2 vendors (JAX and HAR). The 16S rRNA gene sequencing of stool samples distinguished gut microbiota composition between JAX and HAR: *Bacteroidales S24-7* dominant commensals in JAX while a wide range of bacteria belonging to the *Bacteroidetes* phylum in HAR.[105] After vancomycin treatment, bacteria belonging to the *Bacteroidetes* phylum were eliminated in JAX and HAR.[105] Vancomycin treatment did not change the effect of ACT in JAX.[105] However, this antibiotic intervention significantly enhanced the efficacy of ACT in HAR to an extent similar to the treatment effect in JAX.[105] Besides, additional vancomycin administration remarkably increased the abundance and activity of tumor-specific TIL.[105] This transformation to hot tumor was attributed to accumulated $CD8\alpha^+$ DC and IL-12 in peripheral circulation, as well as concurrent enhanced Th1-skewed immune response.[105]

## Putative Mechanisms by Which the Gut Microbiota May Regulate the Effect of Anticancer Immunotherapy

Anticancer immunity is described by a model called the cancer-immunity cycle. Tumor-derived antigens initiate the immune response.[60] After capture and presentation of antigen presentation cells, naïve T cells are primed and activated in peripheral lymphatic organs.[60] Then, primed T cells migrate and infiltrate the tumor bed.[60] Following the recognition of tumor antigens, activated T cells kill tumor cells.[60] During tumor initiation and progression, one or more steps in cancer-immunity cycle are impaired.[60] Anticancer immunotherapy is developed to unleash the exhausted T cells and restore anticancer immune response.[106] Based on the cancer-immunity cycle, immunotherapy could compensate for one or multiple undermined anticancer immune procedures. However, as a cascade reaction, the actual effect of immunotherapy is limited by its upstream or downstream factors such as systemic cytokine repertoire, the cross-presentation of antigen presentation cell, as well as the inhibitory components in the tumor immune microenvironment.[107] Gut microbiota could regulate a broad range of immune effectors, especially DC. As the core of antigen presentation and T cell activation, the function of DC is the determinant of immune surveillance and immune clearance. Some bacteria such as *Bifidobacterium* could enhance the function of DC by promoting DC maturation, upregulating cytokine secretion, stimulating DC-IL-12-Th1-skewing immune response, as well as facilitating the activation and survival of tumor-specific T cells.[99] The cross-talk between gut microbiota and DC in PP not only induces local immune response in

gut mucosa but also regulates systemic immune response by the peripheral circulation.[25] Locally generated cytokines and active DCs enter into circulation that could provide a favorable immune tone and synergize with concurrent anticancer immunotherapy.[25,91] Besides this nonspecific immune augmentation, partial bacteria antigen–loaded DCs might lead to molecular mimicry and eliminate tumor cells sharing similar antigen repertoire with gut microbiota.[91]

## Clinical Application of Gut Microbiota in Immunotherapy

Motivated by the encouraging results of preclinical studies, multiple clinical trials investigating the influence of gut microbiota on immune cancer efficacy are ongoing. In 2019, Jin et al reported the data from non–small cell lung cancer patients undergoing nivolumab therapy (patients were enrolled from CheckMate 078 and CheckMate 870 studies).[108] By analyzing the fecal samples of patients before and after anti-PD-1 therapy via 16S rRNA gene sequencing, researchers found that patients with higher diversity of gut microbiota possessed prolonged PFS compared with ones with lower diversity of gut microbiota.[108] Moreover, patients responding to nivolumab therapy possessed higher diversity of gut microbiota at baseline, which sustained stable composition during treatment.[108] Composition difference analysis between responder group and nonresponder group showed that bacteria such as *Alistipes putredinis, B longum*, and *Prevotella copri* were significantly enriched in responders, while unclassified *Ruminococcus* were enriched in nonresponders.[108] Besides, given that antibiotics could reshape the composition of gut microbiota that further interferes the effect of immunotherapy, the relationship between antibiotic-associated dysbiosis and immunotherapy is another hot topic.[109] Elkrief et al found that antibiotic treatment before immunotherapy such as anti-PD-1/PD-L1 and anti-CTLA-4 was an independent risk factor for worse PFS (hazard ratio = 0.32, 95% confidence interval = 0.13-0.83, $P = .02$).[109] Patients receiving antibiotic treatment prior to immunotherapy exhibited lower possibility to effectively respond to immunotherapy (objective response rate of antibiotic group vs control group = 0% vs 34%) and improved prognosis (PFS of antibiotic group vs control group = 0.28, 95% confidence interval = 0.10-0.76, $P = .01$).[109]

Apart from utilizing gut microbiota to predict the efficacy of immunotherapy, some clinical studies focused on how to modulate the composition of gut microbiota to overcome anti-PD-1/PD-L1 resistance. NCT03341143 is a single-center phase 2 trial interrogating the efficacy of fecal microbiota transplant (FMT) plus pembrolizumab in melanoma patients resistant to anti-PD-1 therapy.[110] In this phase 2 trial, FMT was conducted as following procedures: collecting stool from tested donors, mixing with saline or other solutions, then straining and infusing into colon by colonoscopy.[110] NCT03341143 is ongoing, and the results of this study have not been reported.[110] Meanwhile, NCT03595683 (phase 2 trial) evaluated the treatment effect of pembrolizumab with additional EDP1503 (an orally delivered monoclonal microbiota product).[111] In this trial, 70 melanoma patients were involved and received pembrolizumab treatment (200 mg/3 weeks) and concurrent EDP1503 ($\geq 15 \times 10^{10}$ colony-forming units/day).[111] Moreover, the treatment effect of combination therapy of other additional oral microbiome interventions such as SER-401 (NCT03817125) and ICI are under investigation.[112]

Although a myriad of preclinical studies demonstrated that gut microbiota regulated host systemic immune response, modulated immunotherapy efficacy, and affected treatment-induced adverse effects, the regulatory function of certain commensal bacteria still needs further investigation, especially for the extrapolation from the mouse model to humans. The results of these ongoing studies might provide more stable evidence to support the feasibility of enhancing immunotherapy effect by modulating gut microbiota composition. However, it is notable that original gut mucosa commensals interfere with the colonization of supplemental probiotics.[113] The extent of resistance to probiotics colonization is heterogeneous among populations and could be influenced by baseline commensal status.[113] Therefore, patient's commensal background should be taken into consideration for manipulating gut microbiota by interventions such as fecal transplantation. Notably, in 2019, it has been reported that 2 patients receiving FMT treatment developed invasive infections caused by multidrug-resistant organisms and one of the patients died. It is necessary to keep alert to FMT therapy–induced adverse events in further clinical investigation.

## Conclusion

Gut microbiota has a substantial influence on host immune response and modulates multiple steps of cancer-immunity cycle including antigen presentation, T cell priming, and activation. Manipulating gut microbiota to induce the formation of systemically immunologic tone is helpful to enhance effect and overcome resistance in immunotherapy. Identifying favorable bacteria and exploring feasible approaches to manipulating gut microbiota would be meaningful to cancer immunotherapy.

### Author Contributions

MY performed the selection of literature, drafted the manuscript, and prepared the figures. DJ, SQ, and QC collected the related references and participated in discussion. KW and AL designed this review and revised the manuscript. All authors contributed

to this manuscript. All authors read and approved the final manuscript.

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# Effect of Selected Stilbenoids on Human Fecal Microbiota

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**Abstract:** Dietary phenolics or polyphenols are mostly metabolized by the human gut microbiota. These metabolites appear to confer the beneficial health effects attributed to phenolics. Microbial composition affects the type of metabolites produced. Reciprocally, phenolics modulate microbial composition. Understanding this relationship could be used to positively impact health by phenolic supplementation and thus create favorable colonic conditions. This study explored the effect of six stilbenoids (batatasin III, oxyresveratrol, piceatannol, pinostilbene, resveratrol, thunalbene) on the gut microbiota composition. Stilbenoids were anaerobically fermented with fecal bacteria from four donors, samples were collected at 0 and 24 h, and effects on the microbiota were assessed by 16S rRNA gene sequencing. Statistical tests identified affected microbes at three taxonomic levels. Observed microbial composition modulation by stilbenoids included a decrease in the Firmicutes to Bacteroidetes ratio, a decrease in the relative abundance of strains from the genus *Clostridium*, and effects on the family *Lachnospiraceae*. A frequently observed effect was a further decrease of the relative abundance when compared to the control. An opposite effect to the control was observed for *Faecalibacterium prausnitzii*, whose relative abundance increased. Observed effects were more frequently attributed to resveratrol and piceatannol, followed by thunalbene and batatasin III.

**Keywords:** phenolics; polyphenols; stilbenoids; human gut microbiota; 16S rRNA gene sequencing; batatasin III; oxyresveratrol; piceatannol; pinostilbene; resveratrol; thunalbene; fermentation; human colon model; *Lachnospiraceae*; Firmicutes; Bacteroidetes; *Clostridium*; *Faecalibacterium prausnitzii*

## 1. Introduction

Stilbenoids are a subclass of plant-derived phenolic compounds often consumed in the diet as components from red grapes, peanuts, certain berries, and many others. Their average dietary intake is 1 g/day [1–3]. The most well studied stilbenoid is resveratrol, which came into the spotlight with the so-called French paradox, where it was attributed in reducing coronary heart disease mortality among the sample population despite the strong presence of risk factors [4,5]. Further studies have attributed many other potential health benefits to resveratrol, as well as to various other phenolics, such as potent

antioxidant activity, cardio-protection, neuroprotection, anti-inflammatory effects, cancer prevention, and others [4].

In plants, phenolics are usually conjugated to sugars, organic acids, and macromolecules (e.g., dietary fiber and proteins) and most of them are not properly released and absorbed in the small intestine, reaching the colon for further microbial fermentation; at colonic level, they are fermented by the resident gut microbiota (GM) [6]. It is the resulting metabolites that are attributed the health benefits as bioactive compounds. Evidence shows that 90–95% of ingested dietary phenolics, usually in their glycosylated form, are not absorbed in the upper part of the digestive tract. Most of them reach the colon, where the GM metabolize them into lower molecular weight-phenolic compounds, such as phenolic acids, that can be more easily absorbed by intestinal epithelial cells and enter the liver for further biotransformation or systemic circulation [7–13]. These microbial bio-transformations are grouped into three major catabolic processes: hydrolysis (O-deglycosylations and ester hydrolysis), cleavage (C-ring cleavage; delactonization; demethylation), and reductions (dehydroxylation and double bond reduction) [14]. Reciprocal to these bio-transformations by the GM, phenolics appear to modulate the GM composition by favoring/disfavoring certain microbial strains, thus establishing a two-way relationship between the GM and phenolics [6,15–18]. The undigested phenolics, along with diet-independent substrates like endogenous host secretions, are the main substrates of gut bacterial metabolism, and may affect the GM in a similar manner as prebiotics, shape microbial composition by antimicrobial action, and/or influence bacterial adhesion [2,19–24]. For example, chlorogenic acid, resveratrol, catechin, and certain quercetin derivatives have exhibited prebiotic-like effects by increasing the proportional representation of *Bifidobacterium* strains [2,7,25–27]. Antimicrobial action has been shown by inoculation with resveratrol and certain ellagitannins by inhibiting the growth of several *Clostridia* species [2,12,17,28]. Bacterial adhesion effects by procyanidin and chlorogenic acid have been noticed through adhesion enhancement of certain *Lactobacillus* strains to intestinal epithelial cells [23,24].

To our knowledge, except for resveratrol and a few studies with piceatannol, both of which are well-recognized plant-derived phenolics, there is not much information regarding the effects of stilbenes on the GM. Other than a 2016 study on the effects of repeated stilbenoid administration on the GM, the rest have mostly focused on evaluating a single dose effect, mainly on culturable microbial strains. The findings from the former showed a strong change in the GM composition after application of resveratrol and viniferin, especially in the enrichment of the order Enterobacteriales, and a decrease of Bifidobacteriales [29]. Observations from the single dose studies showed changes in the GM composition; for example, increases for species *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* by resveratrol, and in the genus *Lactobacillus* by piceatannol [2,7,8,27,30–34].

The objective of this study was to assess the effect of six stilbenoid phenolics (batatasin III (Bat), oxyresveratrol (Oxy), *trans*-resveratrol (Res), piceatannol (Pic), pinostilbene (Pino), and thunalbene (Thu); the corresponding chemical structures are given in Figure 1) on the GM at dietary relevant concentrations. Using an in vitro fecal fermentation (FFM) system, these stilbenoids were fermented with human fecal bacteria from four donors. Effects on the GM composition were based on 16S rRNA gene sequencing results.

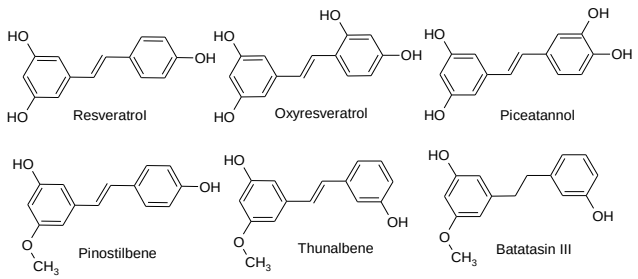


**Figure 1.** Molecular structures of stilbenoids studied. All stilbenoids have a $C_6$-$C_2$-$C_6$ structure.

## 2. Results and Discussion

### *2.1. Firmicutes to Bacteroidetes (F/B) Ratio*

The most abundant phyla in human gut microbiota are Firmicutes and Bacteroidetes, which often account for more than 90% of the total gut microbiota [35]. However, that was not the case in this study. Firmicutes were the most abundant, followed by Actinobacteria, with Bacteroidetes coming in at either fourth or fifth place depending on the donor. One possibility may be that one of the kits used during processing may have been more sensitive to phyla other than Bacteroidetes, or perhaps these bacteria progress to a higher relative abundance during *in vitro* cultivation compared to what would normally be found in stool alone. Nevertheless, the ratio of these two phyla can still be evaluated.

An increased F/B ratio in both human and mouse gut microbiota has consistently been associated with higher obesity and disease occurrence [36,37]. Resveratrol has been previously shown to decrease this ratio [2,27,38], and our findings support this. Similarly, the other tested stilbenoids also decreased the F/B ratio as can be seen in Figure 2. Res, Bat, and Thu reached lower ratios (61 ± 23, 49 ± 22, 96 ± 53 respectively) than the control at 24 h (121 ± 73). Interestingly, Pino showed an increase (227 ± 127), while Pic stayed approximately equal (131 ± 98) to the control at 24 h. The response is a result of a decrease in the relative abundance of Firmicutes and an increase of Bacteroidetes, which is consistent with findings from other studies [2,7,27]. For Firmicutes, after treatment with all tested stilbenoids, the relative abundance decrease (−2.9% ± 0.03%) was lower than the control at 24 h (−4.6% ± 0.03%), with the least decrease observed under Oxy and Pino (−1.5% ± 0.03% and −0.7% ± 0.02%, respectively). For Bacteroidetes, after treatment with all tested stilbenoids except for Pino (51.0.2% ± 0.00%), the growth in relative abundance (Bat 278.0% ± 0.02%; Oxy 198.1% ± 0.00%; Pic 86.0% ± 0.05%; Res 195.6% ± 0.04%; Thu 300.3% ± 0.01%) was greater than that of the control at 24 h (68.0% ± 0.04%).

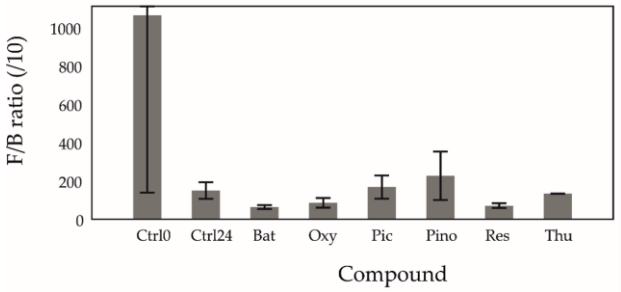


**Figure 2.** Mean Firmicutes/Bacteroidetes ratio (/10) in fermentations. Error bars represent the 95% CI. Ctrl0 = control at 0 h; Ctrl24 = control at 24 h; Bat = batatasin III; Oxy = oxyresveratrol; Pic = piceatannol; Pino = pinostilbene; Res = *trans*-resveratrol; Thu = thunalbene. All stilbenoids at 24 h.

### *2.2. Most and Least Abundant Species*

A total of 230 bacterial species entities were detected in the tested fecal samples. This number includes unidentified species that could only be categorized as part of a higher taxonomic level. For example, an unidentified species, from an unidentified genus, that belongs to the *Clostridiaceae* family. The lowest detected relative abundance was 0.00047% for an unidentified species of the *Christensenella* genus.

The five species with the highest relative abundance per each of the tested samples were identified. These accounted for 53% to 66% of the total relative abundance and, in total, comprised 11 distinct species (Table 1). Therefore, there appears to be certain consistency, and not much variability, among the most abundant taxa.

**Table 1.** The most abundant species obtained by identifying the five species with the highest relative abundance for Control 0 h and 24 h, and per each of the six tested stilbenoid samples at 24 h. Gen. = unnamed genus, sp. = unnamed species.

| Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|
| Actinobacteria | Actinobacteria | Bifidobacteriales | *Bifidobacteriaceae* | *Bifidobacterium* | *sp.* |
| Firmicutes | Bacilli | Lactobacillales | *Streptococcaceae* | *Streptococcus* | *sp.* |
| | Clostridia | Clostridiales | *Lachnospiraceae* | *Blautia* | *sp.* |
| | | | | *Gen.* | *sp.* |
| | | | | *Gen.* | *sp.* |
| | | | *Ruminococcaceae* | *Faecalibacterium* | *prausnitzii* |
| | | | | *Ruminococcus* | *sp.* |
| | | | | *Gen.* | *sp.* |
| | | | | *Gen.* | *sp.* |
| | | | *Unnamed* | *Gen.* | *sp.* |
| Proteobacteria | Gammaproteobacteria | Enterobacteriales | *Enterobacteriaceae* | *Gen.* | *sp.* |

Focusing on the inverse, in the five species with the least relative abundance, there is less consistency and greater variability since it comprised 27 distinct species (Appendix A, Table A1). It's important not to ignore the least abundant species since their low abundance may not necessarily correlate with the importance of their function. As stated in Cueva et al., the microorganisms present in smaller quantities, but developing specific functions, could be the key to understanding the individual response to consumption of bioactive compounds (i.e., phenolics). Some metabolic functions seem to be achieved by a wide variety of species, while other functions are only done by a specific few [15]. For example, *Ruminococcus bromii*, identified within the 27 species, has been noted to be a butyrate (a short-chain fatty acid) producer, which is a function that appears to be found in fewer species than those for acetate [39].

### *2.3. Changes in Relative Abundance (Phylum, Family, Species)*

Both parametric and non-parametric statistical tests were used to identify taxa of interest at the phylum, family, and species level based on two comparisons. The statistical tests were used as a tool to identify potential significantly affected taxa, and should not be interpreted as a portrayal of definite statistical significance (for those with p values in the range) due to the small sample size (four donors). The identified taxa reported $p < 0.075$ for at least one $p$ value (paired sample t-test and/or Wilcoxon signed-rank test) for both comparisons 1 and 2. Comparison 1 used as a baseline the relative abundance of the 24 h control, and compared this value to each of the six stilbenoid fermentations. Comparison 2 used as a baseline the magnitude of change (growth or decline) in relative abundance between Control 0 h and Control 24 h, and compared this value to the magnitude of change between Control 0 h and each of the six stilbenoid fermentations.

Figure 3 displays these identified taxa in the form of a phylogenetic tree sorted by phylogenetic distance. The corresponding $p$ values are listed in Appendix A, Table A2, and the corresponding relative abundance box plots are shown in Figure 4. Each comparison (1&2) is shown separately in Appendix A, Tables A3 and A4, and list additional taxa. Clustered bar graphs of bacterial composition at the phylum and family levels can be seen in Appendix A, Figures A1 and A2. Table 2 displays how our study compares to findings and observations from other studies regarding the effect of the selected stilbenoids on a specific taxon.

#### 2.3.1. Decrease in Relative Abundance

A decrease in relative abundance was observed for several taxa under some of the tested stilbenoids. The most frequently observed response was a further decrease of the relative abundance

of a specific taxon as compared to the 24 h control by either Res, Pic or Thu. For example, for *Clostridium sp.* there was a decrease of −54.2% ± 28.8% for Ctrl24, while the decrease caused by Pic and Thu were of a greater magnitude, −62.9% ± 28.0% (t(3) = 3.960, p = 0.029) and −79.3% ± 22.6% (t(3) = 3.901, p = 0.030), respectively. Similar responses were observed, albeit at different magnitudes, for family *Lachnospiraceae,* and species *Coprococcus sp.*, *Collinsella aerofaciens,* and *Lachnospiraceae Gen. sp.* At the genus level, *Clostridium* decreased under all tested stilbenoids in our study. Previous findings, as listed in Table 2, observed that several species from the genus *Clostridium*, which includes both commensal and deleterious species, had been shown to decrease with resveratrol [2,12].

A second observed response was a decrease in relative abundance while the 24 h control increased. This was observed by three species, *Ruminococcus sp.* (−3.2% ± 69.1%, t(2) = 4.448, p = 0.047 under Bat; −7.0% ± 69.4%, t(3) = 8.253,p = 0.004 under Pic; −41.1% ± 50.9%, t(3) = 1.953, p = 0.146 under Thu), *Ruminococcus sp.* (−3.3% ± 12.7%, t(3) = 3.947, p = 0.029 under Res), and *Coriobacteriaceae Gen. sp.* (−0.9% ± 94.2%, t(2) = 6.272, p = 0.024 under Oxy; −3.7% ± 90.6%, t(3) = 3.261,p = 0.047 under Pic; −39.2% ± 10.0%, t(3) = 1.726, p = 0.183 under Thu), while they increased in the 24 h control (27.8% ± 80.6%, 32.2% ± 68.5%, 15.5% ± 20.8%, respectively). Regarding *Ruminococcus,* this may not be a favorable response according to recent research that points to a high proportion of long-chain dietary fibers degraders, butyrate producing bacteria such as *Ruminococcus, Eubacterium, and Bifidobacterium* as being part of healthy gut microbiota [11,40–42]. The *Ruminococcus* genus has previously been identified as one of the three taxa, besides *Bacteroides* and *Prevotella*, that define the enterotype concept, which could help in explaining variability in responders/non-responders in intervention studies [43]. In regards to *Coriobacteriaceae*, it has been noted that many species that metabolize phenolics belong to this family, however, its potential health implications are still poorly understood [6]. Nevertheless, one important aspect of this family is that all identified *S*-equol-producing bacteria, except for the genus *Lactococcus,* belong to it [44,45].

A third observed response was a decrease in relative abundance while the 24 h control also decreased, but with a larger magnitude. This was observed for *Blautia obeum*, which was recently reclassified, its former name being *Ruminococcus obeum* [46]. *Blautia* has been considered one of the major representatives of the Firmicutes phylum due to its relatively high abundance [15]. This species experienced a decrease in relative abundance by thunalbene (−5.6% ± 32.1%, (t(3) = 3.763, p = 0.033), but at a lower magnitude than the control at 24 h (−29.8% ± 35.6%). A decrease of *Blautia,* at the genus level, was also reported in a study conducted on mice fed a phenolic-enriched tomato diet, as well as in a study of human fecal fermentation study after consumption of phenolics from tart cherries [26,31]. These findings, along with our study, suggest that certain phenolics may cause a decrease in this genus, but at a lesser magnitude than without it. This taxon also appears to be a butyrate-producing microbe whose reduction has been correlated with decreased production of butyrate [47].

Eight of the identified taxa belonged to the family *Lachnospiraceae*. There was no consistent response from the tested stilbenoids within this family however, the most frequent response was a decrease in relative abundance. This decrease was also observed in a study where rats were supplemented with the stilbenoid pterostilbene in their diet. In that study, *Lachnospiraceae* was significantly reduced in each tested group when compared to baseline levels [33].

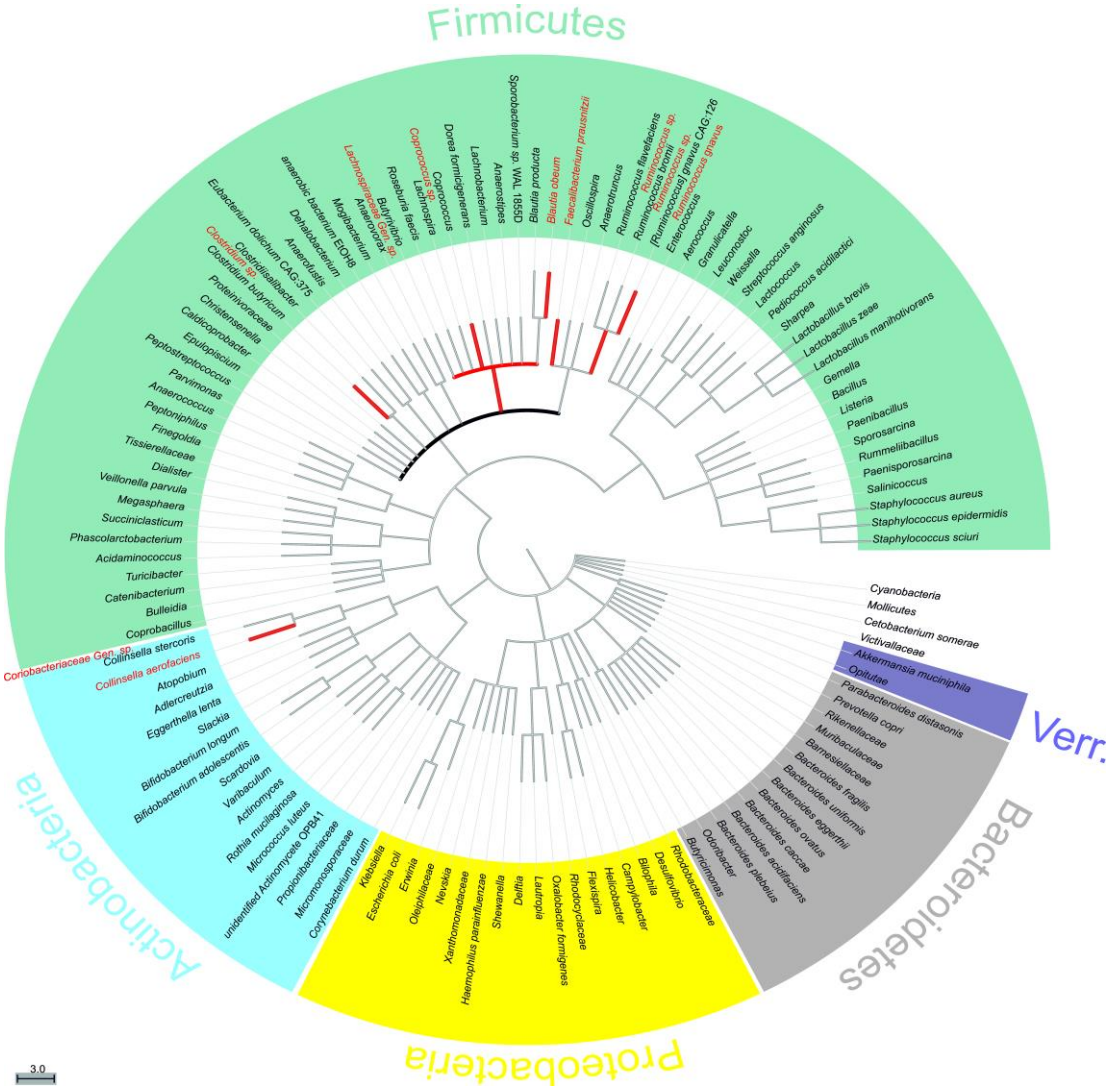


**Figure 3.** Phylogenetic tree of all identified bacterial entitites. The tree is sorted by phylogenetic distance, therefore the closer they are on the tree, the closer they are genetically [41,42]. Taxa shown in red displayed $p < 0.075$ for at least one p value (Paired sample t-test and/or Wilcoxon signed-rank test) for both comparisons 1 and 2. Bolded black line refers to family Lachnospiraceae. Verr. = Verrucomicrobia. Gen. = unnamed genus, sp. = unnamed species.

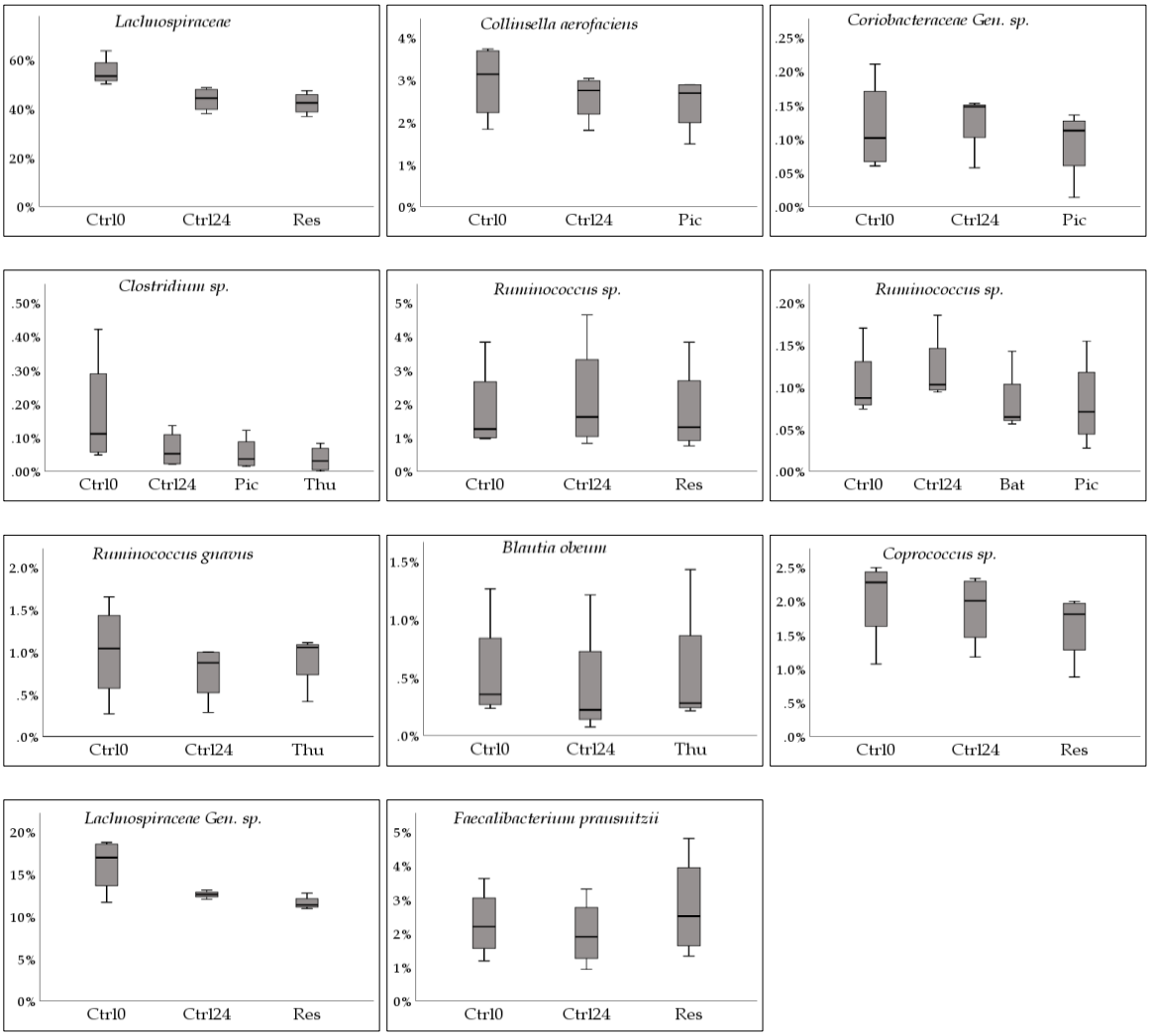


**Figure 4.** Box plots corresponding to the identified species and stilbenoids in Table A2. The y-axis displays relative abundance (%). The x-axis shows Ctrl0, control at 0 h; Ctrl24, control at 24 h; as well as the stilbenoid(s) corresponding to the observation (Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene (all stilbenoids at 24 h). Gen. = unnamed genus, sp. = unnamed species.

### 2.3.2. Increase in Relative Abundance

An increase in relative abundance with no change in the 24 h control was observed for *Faecalibacterium prausnitzii* under Res (36.6% ± 88.0%, t(3) = −2.806, p = 0.068 under Res), 24 h control (−0.5% ± 62.5%). This species has been previously identified as a butyrate producing bacterium and is regarded as being beneficial. Butyrate production appears to be key in maintaining the colonic epithelium by inducing proliferation of healthy colonocytes. Fiber-poor diets, such as the one our donors were subject to prior to sample donation, have been associated with low butyrate production. One study showed a strong positive correlation between the proportion of *F. prausnitzii* and that of butyrate in individuals on a normal diet, and the reduction in *F. prausnitzii* on switching to a fiber-free or fiber-supplemented diet correlated with the reduction in fecal butyrate [47,48]. The gut epithelium is the main body site for butyrate sequestration, and low butyrate production has been connected to inflammatory diseases such as ulcerative colitis [39,49]. Unlike acetate producing bacteria, which are widely distributed, there appear to be fewer butyrate producing bacteria such as *S. prausnitzii*, *E. rectale*, *E. hallii*, and *R. bromii* [38]. It was observed to increase in plant-based, fiber-rich, diets, thus,

stilbenoids being phytochemicals, were expected to increase their abundance. Our findings support this with resveratrol.

An increase in relative abundance with a decrease in the 24 h control was observed for *Ruminococcus gnavus* under Thu (8.2% ± 40.6%, t(3) = −2.244, p = 0.111 under Thu), 24 h control (−12.9% ± 30.7%). The observed p value, along with the box plot in Figure 4, show that *R. gnavus'* increase was not as pronounced as that of *F. prausnitzii*. Both of these taxa tend to be quite reduced in inflammatory bowel diseases such as Crohn's disease [50,51].

Although it was detected in only one of our donors, *Akkermansia muciniphila* was observed to be enhanced by resveratrol. This species has been previously observed to be enhanced by pterostilbene, which has shown to exhibit similar cellular effects to resveratrol. One of these is that both phenolics have been hypothesized to mimic caloric restriction effects at the molecular level, thus modifying the gut microbiota, especially enhancing *A. muciniphila* [33].

These findings emphasize the importance of trying to get to the lowest possible taxonomic level to better characterize the gut microbiota. As can be seen from our study, species within the same family level are not all uniform in their responses. Higher taxonomic levels are quite useful, and can make experiments and data processing much more manageable; however, care must be taken in generalizing for every member of a taxon.

Whether the microbiota response is a decrease or an increase in relative abundance, effects are more frequently attributed to resveratrol and piceatannol, followed by thunalbene and batatasin III. This difference may be related to their chemical moieties. All stilbenoids share a basic C6-C2-C6 structure, differing only in the presence or absence of a C-C double bond on -C2-, and on the type and position of functional groups, mainly hydroxyl (-OH) and o-methoxyl (-$OCH_3$) groups on the aromatic rings. In phenolics, -OH groups play an important role on their bioactivity, and their substitution by -$OCH_3$ groups has been shown to reduce their bioactivity [52–54]. -OH groups are good hydrogen donors, are considered very reactive and potent radical scavengers, are key in the general antioxidant mechanism of resveratrol, and it has been shown that phenolics with more -OH groups exhibit higher capacity for enzyme inhibition than those with -$OCH_3$ groups [53–57]. Enzyme inhibition capacity has also been shown to be affected by hydrogenation of the C-C double bond on -C2-, which decreased enzyme inhibition [54,58–60]. This suggests that phenolics with -OH moieties and C-C double bond on -C2- may be more bioactive than those with -$OCH_3$ moieties and lacking a C-C double bond on -C2-. Resveratrol and Piceatannol have three and four -OH groups respectively, as well as a C-C double-bond on -C2-. They were the two stilbenoids that were most frequently attributed effects on the GM in this study. These were followed by thunalbene, which is O-methylated and has a C-C double bond on -C2-, and by batatasin III, which is O-methylated and lacks a C-C double bond on -C2-. Regarding demethylation, a recent study reported a demethylated colonic metabolite of the phenolic curcumin by *Blautia sp.* MRG-PMF1 [61]. Thunalbene is O-methylated and, as reported earlier, *Blautia sp.* experienced a decrease in relative abundance under thunalbene, but at a lower magnitude than that of the control. Regarding C-C double bond reduction, Bode et al. showed that *Slackia equalifaciens* and *Adlercreutzia equolifaciens* were able to metabolize resveratrol to dihydroresveratrol by reduction of the C-C double bond, but could not identify any bacteria for the -OH cleavage that produced two other metabolites [8]. Reduction of the C-C double bond by GM has also been shown for other phenolics such as isoflavones and hydroxycinnamates, while -OH cleavage for lignans and phenolic acids [19,62–64]. How chemical moieties affect metabolite production by microbial strains and bioactivities such as antioxidant activity, enzyme inhibition, quorum sensing, and others is outside the scope of our study; nevertheless, it's an important avenue for ongoing and future research.

The interpretation of the results from GM studies such as this one should take into consideration the concept of inter-individual variability. This concept is well known in the literature, the most well-known example being the difference between individuals whose microbiota are either producers or non-producers of the *S*-equol phytoestrogen. Oral administration of *S*-equol results in improvement of certain cardiovascular disease biomarkers, but only on those who are producers [20,47]. Although

our sample size is small, differences among donor GM composition can be visualized in Figures A1 and A2. Donor D2, for example, appears to have a very atypical microbial composition when compared to the other three donors.

**Table 2.** Observations from previous studies regarding the effect of select stilbenoids on specific taxa compared to observations in this study [2,7,8,27,30–34,65]. From the literature, ↑ or ↓ indicate a reported abundance increase or decrease of the strain. From this study, S, NS, Un, ND, signify, respectively, supported, not supported, undefined, not detected. Gen. = unnamed genus, sp. = unnamed species.

| Stilbenoid | Effect | Phylum | Family | Genus | Species | | Notes |
|---|---|---|---|---|---|---|---|
| Resveratrol | ↑ | Actinobacteria | *Bifidobacteriaceae* | *Bifidobacterium* | *sp.* | NS | |
| | | Firmicutes | *Clostridiaceae* | *Clostridium* | *XB90* | S | |
| | | | | *Faecalibacterium* | *prausnitzii* | S | Won't grow without acetate in pure culture. |
| | | | *Lactobacillaceae* | *Lactobacillus* | *sp.* | Un. | |
| | | Bacteroidetes | *Tannerellaceae* | *Parabacteroides* | *distansonis* | NS | Only detected in one donor. |
| | ↓ | Firmicutes | *Clostridiaceae* | *Clostridium* | *aldenense* | S | |
| | | | | | *C9* | S | Species not identified, responsesignificn at genus level. |
| | | | | | *hathewayi* | S | |
| | | | | | *MLG661* | S | |
| | | | *Enterococcaceae* | *Enterococcus* | *faecalis* | ND | |
| | | Proteobacteria | *Gracilibacteraceae* | *Gracilibacter* | *thermotolerans* | ND | |
| | | | *Enterobacteriaceae* | *Proteus* | *mirabilis* | ND | |
| | | Firmicutes to Bacteroidetes (F/B) ratio | | | | S | |
| | Other | Actinobacteria | *Coriobacteriaceae* | *Slackia* | *equolifaciens* | Other | Dihydroresveratrol producers. Identified at genus level only. Slackia's abundance highest for Res, and not detectable at Ctrl0. Adlercreutzia's abundance highest for Ctrl24, and lowest for Ctrl0. |
| | | | | *Adlercreutzia* | *equolifaciens* | Other | |
| Phenolic mix, includes Resveratrol | ↑ | Verrucomicrobia | *Verrucomicrobiaceae* | *Akkermansia* | *muciniphila* | S | Mice study. Detected in one of our donors. |
| | ↓ | Firmicutes | *Lachnospiraceae* | *Blautia* | *sp.* | Un. | Mice study. |
| | | | *Ruminococcaceae* | *Oscillospira* | *sp.* | S | Mice study. Has never been cultured, but always detected. |
| Piceatannol | ↑ | Firmicutes | *Lactobacillaceae* | *Lactobacillus* | *sp.* | NS | Mice study. |
| | | | *Unnamed* | *Gen.* | *sp.* | NS | Mice study. |
| | ↓ | Bacteroidetes | *Unnamed* | *Gen.* | *sp.* | NS | Mice study. Decrease was observed, but at a lower magnitude than Ctrl24. |
| | Other | | *Bacteroidaceae* | *Gen.* | *sp.* | S | Mice study. Abundance change. |
| Fiber | ↑ | Bacteroidetes | *Prevotellaceae* | *Prevotella* | *sp.* | S | Stilbenoids associated with fiber-containing food. |
| Plant-based diet | | Firmicutes | *Clostridiaceae* | *Faecalibacterium* | *prausnitzii* | S | Saccharolytic microbes. |
| | | | *Lachnospiraceae* | *Roseburia* | *sp.* | NS | Saccharolytic microbes. |
| | ↓ | Proteobacteria | *Desulfovibrionaceae* | *Bilophila* | *sp.* | ND | Putrefactive microbes. Less abundance expected in a plant-based diet. |
| | | Bacteroidetes | *Bacteroidaceae* | *Bacteroides* | *sp.* | NS | Putrefactive microbes. Less abundance expected in a plant-based diet. |

## 3. Materials and Methods

### 3.1. Study Design

Using an in vitro fecal fermentation (FFM) system, a set of six stilbenoid phenolics were fermented in vials via inoculation with human fecal bacteria obtained from four donors. The vials were sampled at 0 hour (0 h) and 24 h (24 h) time points, and the effect of the stilbenoids on human GM was assessed by 16S rRNA gene sequencing. Both parametric and non-parametric statistical tests were used to identify potentially affected strains at the phylum, family, and species taxonomic levels.

### 3.2. Donors and Ethics Statement

The fecal samples originated from four volunteer donors, all of whom consented for their samples to be used for research purposes by signing a consent form. The ethical agreement for stool collection was obtained by the ethical committee (ZEK/22/09/2017) of the Czech University of Life Sciences in Prague. The donors were two males and two females ages 23, 28 (Donors 1 and 3) and 26, 29 (Donors 2 and 4) respectively. Their respective body mass index (BMI) were 23.0, 24.7, 26.0, and 26.5. To reduce potential interference from other dietary phenolics, all donors followed a low-phenolic diet for at least 48 hours prior to providing the fecal sample. Also, none had taken any antibiotics for at least 6 months prior to sampling. They described themselves as being in good health, and none reported any chronic conditions or diseases. They followed an omnivorous diet in their daily life. Females were neither pregnant nor lactating. The samples were collected in October and November 2016, at the Czech University of Life Sciences in Prague, Czech Republic.

### *3.3. In vitro Fecal Fermentation (FFM) System*

#### 3.3.1. Standard Compounds and Chemicals

The chemicals used for preparation of the fermentation medium were obtained from Merck (Darmstadt, Germany). The stilbenoids batatasin III, piceatannol, thunalbene, and pinostilbene were purchased from ChemFaces (Wuhan, China) in 98% purity; *trans*-resveratrol, oxyresveratrol were obtained from Merck in 98% purity. Standards were prepared as 1% methanol/formic acid. Methanol and ethyl acetate were of analytical grade and purchased from VWR Chemicals (Stribrna Skalice, Czech Republic). Dimethyl sulfoxide (DMSO) was obtained from VWR Chemicals. Formic acid was obtained from Fisher Scientific (Merelbeke, Belgium) in >98% purity. Ultra-pure water (MilliQ) was obtained from a Millipore system (Bedford, MA, USA).

#### 3.3.2. Fermentation Medium

Fermentation medium was prepared from the following solutions based on previous fecal fermentation studies [66–68]. Micromineral solution was prepared from 2.64 g $CaCl_2$, 2 g $MnCl_2$, 0.2 g $CoCl_2$, 1.6 g $FeCl_3$, and up to 20 mL distilled water. Macromineral solution was prepared from 7.14 g of $Na_2HPO_4$, 6.2 g $KH_2PO_4$, 0.6 g $MgSO_4$, and up to 1 L distilled water. Carbonate buffer was made of 1 g $NH_4HCO_3$, 8.75 g $NaHCO_3$, and distilled water up to 250 mL (stored max. 1 month). The fermentation medium was prepared from 225 mL distilled water and 1.125 g of tryptone, 56.25 µL of micromineral solution, 112.5 mL of $CO_3$ buffer, 112.5 mL of macromineral solution, and 562.5 µL of 0.1% resazurin solution.

#### 3.3.3. Phosphate Buffer, Reducing Solution

Sodium phosphate buffer for the preparation of fecal slurries was made of 1.7702 g $KH_2PO_4$ in distilled water (195 mL), and 3.6222 g $Na_2HPO_4$ in 305 mL distilled water (both 1/15 M). Afterwards, the buffer's pH was modified to 7.0 by hydrochloric acid. Reducing solution was prepared from 125 mg cysteine hydrochloride, 0.8 mL 1 M NaOH, 125 mg $Na_2S$ and distilled water up to 20 mL.

#### 3.3.4. Fermentations Using Human Fecal Microbiota

Each tested stilbenoid was dissolved in DMSO to reach a concentration of 10 mg/mL. The fermentation medium and sodium phosphate buffer were boiled and cooled to approximately 37 °C while they were purged with oxygen free nitrogen gas (approx. 30 min). The medium's pH was adjusted to pH 7.0 using HCl. For each vial, 16.8 mL of medium was transferred to the corresponding fermentation bottle and 0.8 mL of reducing solution was added. Per each donor, freshly obtained feces were homogenized in a stomacher bag with the sodium phosphate buffer to make a 32% fecal slurry. This slurry was then filtered through a mesh, from which 2 mL of the resulting filtrate was mixed with the fermentation medium in each of the fermentation bottles. 20 µL of tested compound solution (or DMSO alone for the controls) was also added. The bottles were incubated at 37 °C for 48 hours in a shaking bath at 100 strokes per minute. Four aliquots of fecal suspensions were prepared in 1.5 mL Eppendorf tubes by transferring from 20 mL glass bottles, collected at 0, 2, 4, 8, 24 and 48 h, and stored at −80 °C until further analysis. These timepoints were used for a related metabolomic study. For this particular study, only 0 and 24 timepoints were used.

### *3.4. Microbial Analysis*

#### 3.4.1. DNA Extraction

Bacterial DNA was isolated from the fecal samples according to the manufacturer's instructions using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA). The purified DNA was eluted in 100 µL of elution buffer and stored at −20 °C until further use.

#### 3.4.2. 16.S rDNA amplification: Nested PCR

During this nested PCR, two genes were amplified and targeted by two different pairs of primers in two successive reactions of PCR. The first PCR was done to amplify almost full length bacterial 16S rRNA gene fragments using the universal bacterial primers 616V (5′(5′ AGA GTT TGA TYM TGG CTC 3′) and 630R (5′ CAK AAA GGA GGT GAT CC 3′) [69]. The thermal cycling was carried out with an initial denaturation step of 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min and 30 s; cycling was completed by a final elongation step of 72 °C for 6 min. Using the purified PCR product from the first PCR, the second PCR was performed as described by Fliegerová et al. [70] to amplify the V4-V5 region of the 16S rRNA gene by the primer pair: BactBF (GGATTAGATACCCTGGTAGT) and BactBR (CACGACACGAGCTGACG). The used thermal cycling program was: initial denaturation for 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C and 30 s at 72 °C, ending by final elongation for 5 min at 72 °C. The PCR amplicons (300 bp) were checked at 1.5% agarose electrophoresis (30 min at 100 V), purified by QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands) according to the protocol and quantified by Nanodrop (Thermo Fisher, Waltham, MA, USA).

#### 3.4.3. Semi-conductor Based Next Generation Sequencing

Obtained PCR products were used to prepare libraries for diversity analyses by next generation sequencing (NGS) approach on Personal Genome Machine (Life Technologies, Carlsbad, CA, USA) according to Milani et al. [71]. 200 ng of DNA from each sample was used to prepare sequencing libraries by NEBNext®Fast DNA Library Prep Set kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol. The Ion Xpress Barcode adapters (Thermo Fisher Scientific, Waltham, MA, USA) were used to label each sample. The adaptor ligated libraries were purified and simultaneously size-selected using AMPure XP bead sizing (Beckman Coulter, Brea, CA, USA). The barcoded libraries were pooled in equimolar amount (about 26 pM). The pool of libraries was used to prepare sequencing template by emulsion PCR on Ion Sphere Particles (ISPs) using Ion PGMTM Hi-QTM View OT2 400 Kit (Thermo Fisher Scientific) in Ion OneTouchTM 2 instrument. The enrichment of the template positive ISPs were performed on Ion OneTouchTM ES instrument. The enriched template positive ISPs were then loaded in Ion 316TM Chip v2 BC (Thermo Fisher Scientific). The sequencing was then performed on an Ion Torrent PGM sequencer (Thermo Fisher Scientific, Waltham, MA, USA) using Ion PGMTM Hi-QTM View Sequencing solutions kit (Thermo Fisher Scientific).

#### 3.4.4. Data Analysis

The sequences obtained in FASTQ format were processed by QIIME analyses pipeline [72]. The chimeras were removed by USEARCH tool [73]. Remaining sequences were clustered and identified by performing open-reference OTU picking against the Greengene database [74]. Diversity index analysis and unweighted and weighted UniFrac distance metrics analyses were generated using QIIME and expressed by principle coordinate analysis (PCoA).

### *3.5. Statistical Analysis*

Using SPSS version 25 (IBM Corp., Armonk, NY, USA), both parametric and non-parametric statistical tests were used to identify taxa of interest at the phylum, family, and species level by the following comparisons: (1) Using the relative abundance of the control fermentation with stool samples at 24 h with DMSO only as our baseline for comparison, we identified taxa from the fermentations with stilbenoids (each comparison done separately) that had $p$ values <0.05 for the Paired sample t-test, and/or <0.075 for the Wilcoxon signed-rank test when compared to our baseline. (2) The magnitude of change (growth or decline) in relative abundance between the control fermentation with only stool sample at 0 h (Ctrl0 h) and our control fermentation with samples with DMSO only at 24 h was

calculated, and this value became our baseline for comparison against the magnitude of change from 0 h to 24 h for the fermentations with stilbenoids. Selected taxa had $p$ values <0.05 for the Paired sample t-test, and/or <0.075 for the Wilcoxon signed-rank test. Only 5 stilbenoids were tested. Pinostilbene was excluded since samples for it were only available for two out of the four donors. Similarly, any pair that had $n \leq 2$ was excluded. Since the data was in percent, the magnitude of change was obtained by obtaining the percentage change of the given percentages. Values of 0% at 0 h were excluded, even if they were detectable at higher percentages. This was done due to the ambiguity of whether they were low values that were undetectable or whether they were simply not present.

## 4. Conclusions

From the surveyed literature, none of the tested stilbenoids, other than resveratrol and piceatannol, had been tested on their effect on the human GM. Our findings suggest that the tested stilbenoids, at physiological concentrations of 10 μg/mL, modulate the GM as observed in a fecal fermentation human colon model. Some of these effects are similar to other studies that have also assessed the effects of dietary phenolics on the GM. Some of our observed effects include a decrease in the Firmicutes to Bacteroidetes ratio, a consistent decrease in the relative abundance of strains from the genus *Clostridium*, and responses from several strains from the family *Lachnospiraceae*. A frequently observed effect on the identified taxa was a further decrease of the relative abundance when compared to the control. An opposite effect to the control was observed for *Faecalibacterium prausnitzii*, which, contrary to the control, increased in relative abundance. This strain has been previously considered beneficial for health. Looking at specific stilbenoids, observed responses were more frequently attributed to resveratrol and piceatannol, followed by thunalbene and batatasin III.

The use of 16S rRNA gene sequencing, in combination with a fecal fermentation human colon model, appears to be a very useful tool to characterize the human GM, especially to identify unculturable strains. It is important to note that studies such as this one are expected to increase in precision as the sensitivity of the detection technology, as well as the taxonomical reference databases, are refined and expanded. The tested stilbenoids appear to support the well-observed view of the potential positive impact of phenolics through the modulation of human GM, and thus further studies are recommended to characterize this microbial environment and its function more precisely.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## Appendix A

**Table A1.** 27 least abundant species. Obtained by identifying the five species with the lowest relative abundance for Control 0 and 24 and per each of the six stilbenoids. Gen. = unnamed genus, sp. = unnamed species.

| Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|
| Actinobacteria | Actinobacteria | Bifidobacteriales | *Bifidobacteriaceae* | *Bifidobacterium* | *adolescentis* |
| | | | | | *longum* |
| | Coriobacteriia | Coriobacteriales | *Coriobacteriaceae* | *Eggerthella* | *lenta* |
| | | | | *Gen.* | *sp.* |
| Firmicutes | Bacilli | Bacillales | *Staphylococcaceae* | *Staphylococcus* | *sp.* |
| | | Lactobacillales | *Lactobacillaceae* | *Lactobacillus* | *sp.* |
| | | | *Leuconostocaceae* | *Weissella* | *sp.* |
| | | | *Streptococcaceae* | *Lactococcus* | *sp.* |
| | | | | *Streptococcus* | *sp.* |
| | Clostridia | Clostridiales | *[Mogibacteriaceae]* | *Gen.* | *sp.* |
| | | | *Clostridiaceae* | *Gen.* | *sp.* |
| | | | *Lachnospiraceae* | *[Ruminococcus]* | *gnavus* |
| | | | | *Blautia* | *sp.* |
| | | | | | *sp.* |
| | | | | *Lachnospira* | *sp.* |
| | | | | *Roseburia* | *faecis* |
| | | | | | *sp.* |
| | | | *Ruminococcaceae* | *Ruminococcus* | *bromii* |
| | | | | | *sp.* |
| | | | *Veillonellaceae* | *Dialister* | *sp.* |
| | | | | *Phascolarctobacterium* | *sp.* |
| | | | | *Succiniclasticum* | *sp.* |
| | Erysipelotrichi | Erysipelotrichales | *Erysipelotrichaceae* | *[Eubacterium]* | *biforme* |
| | | | | *Gen.* | *sp.* |

**Table A1.** *Cont.*

| Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|
| Proteobacteria | Betaproteobacteria | Burkholderiales | *Alcaligenaceae* | *Sutterella* | *sp.* |
| | Deltaproteobacteria | Desulfovibrionales | *Desulfovibrionaceae* | *Bilophila* | *sp.* |
| | Gammaproteobacteria | Enterobacteriales | *Enterobacteriaceae* | *Gen.* | *sp.* |

**Table A2.** Taxa that displayed p < 0.075 for at least one *p* value (Paired sample t-test and/or Wilcoxon signed-rank test) for both comparisons 1 and 2. Bolded *p* values are those <0.05 for the t-test, and ≤0.068 for the signed-rank test. Results from pairs with n ≤ 2 were excluded, which includes all pinostilbene samples. See materials and methods section for more details. Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene. *Gen.* = unnamed genus, *sp.* = unnamed species.

| Phylum | Class | Order | Family | Genus | Species | Stilbenoid | Magnitude Change from 0 h to 24 h | | | | | | Rel. Abundance at 24 h | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Mean(%) ± SD | | | df | Paired-T | Wilcoxon | Paired-T | Wilcoxon |
| Firmicutes | Clostridia | Clostridiales | *Lachnospiraceae* | *Collinsella* | *aerofaciens* | Control | −20.1 | ± | 9.7 | | | | | |
| | | | | | | Res | −22.9 | ± | 10.2 | 3 | 0.025 | 0.068 | 0.045 | 0.068 |
| Actinobacteria | Coriobacteriia | Coriobacteriales | *Coriobacteriaceae* | | | Control | −1.0 | ± | 51.5 | | | | | |
| | | | | | | Pic | −6.2 | ± | 51.2 | 3 | 0.075 | 0.068 | 0.097 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | 27.8 | ± | 80.6 | | | | | |
| | | | | | | Pic | −3.7 | ± | 90.6 | 3 | 0.047 | 0.068 | 0.020 | 0.068 |
| | | | *Clostridiaceae* | *Clostridium* | *sp.* | Control | −54.2 | ± | 28.8 | | | | | |
| | | | | | | Pic | −62.9 | ± | 28.0 | 3 | 0.029 | 0.068 | 0.098 | 0.068 |
| | | | | | | Thu | −79.3 | ± | 22.6 | 3 | 0.030 | 0.068 | 0.043 | 0.068 |
| | | | | | *sp.* | Control | 32.2 | ± | 68.5 | | | | | |
| | | | | | | Bat | −3.2 | ± | 69.1 | 2 | 0.047 | 0.109 | 0.004 | 0.109 |
| | | | | | | Pic | −7.0 | ± | 69.4 | 3 | 0.004 | 0.068 | 0.021 | 0.068 |
| | | | | *[Ruminococcus]* | *sp.* | Control | 15.5 | ± | 20.8 | | | | | |
| | | | | | | Res | −3.3 | ± | 12.7 | 3 | 0.029 | 0.068 | 0.110 | 0.068 |
| Firmicutes | Clostridia | Clostridiales | | | *gnavus* | Control | −12.9 | ± | 30.7 | | | | | |
| | | | | | | Thu | 8.2 | ± | 40.6 | 3 | 0.111 | 0.068 | 0.057 | 0.068 |
| | | | *Lachnospiraceae* | *Blautia* | *obeum* | Control | −29.8 | ± | 35.6 | | | | | |
| | | | | | | Thu | −5.6 | ± | 32.1 | 3 | 0.033 | 0.068 | 0.061 | 0.068 |
| | | | | *Coprococcus* | *sp.* | Control | −5.3 | ± | 11.9 | | | | | |
| | | | | | | Res | −19.9 | ± | 3.6 | 3 | 0.063 | 0.068 | 0.030 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | −19.0 | ± | 18.4 | | | | | |
| | | | | | | Res | −25.5 | ± | 16.7 | 3 | 0.041 | 0.068 | 0.040 | 0.068 |
| | | | | *Faecalibacterium* | *prausnitzii* | Control | −0.5 | ± | 62.5 | | | | | |
| | | | | | | Res | 36.6 | ± | 88.0 | 3 | 0.068 | 0.068 | 0.062 | 0.068 |

**Table A3.** Identified taxa based on Comparison 1, which used as a baseline the relative abundance of the 24 h control, and compared that to each of the six stilbenoid fermentations at 24 h. Taxa displayed Paired-T $p$ value <0.05 and/or Wilcoxon Signed Rank $p$ value <0.075. Bolded values are those within these ranges. Gen. = unnamed genus, sp. = unnamed species.

| | | | | | | | | | | | | **Paired-T** | **Wilcoxon** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phylum** | **Class** | **Order** | **Family** | | | **Stilbenoid** | **Mean(%) ± SD** | | | **t** | **df** | **P < 0.05** | **P < 0.075** |
| Actinobacteria | Actinobacteria | Actinomycetales | *Actinomycetaceae* | - | | Control | 0.16 | ± | 0.28 | | | | |
| | | | | | | Pic | 0.11 | ± | 0.20 | 1.352 | 3 | 0.269 | 0.068 |
| | Coriobacteriia | Coriobacteriales | *Coriobacteriaceae* | - | | Control | 3.68 | ± | 0.93 | | | | |
| | | | | | | Pic | 3.48 | ± | 1.12 | 2.114 | 3 | 0.125 | 0.068 |
| Firmicutes | Bacilli | Lactobacillales | *Aerococcaceae* | - | | Control | 0.04 | ± | 0.06 | | | | |
| | | | | | | Res | 0.02 | ± | 0.04 | 1.417 | 3 | 0.252 | 0.068 |
| | | | *Leuconostocaceae* | - | | Control | 0.11 | ± | 0.19 | | | | |
| | | | | | | Res | 0.13 | ± | 0.21 | −1.733 | 3 | 0.182 | 0.068 |
| | Clostridia | Clostridiales | *Lachnospiraceae* | - | | Control | 43.72 | ± | 4.93 | | | | |
| | | | | | | Res | 42.15 | ± | 4.53 | 3.312 | 3 | 0.045 | 0.068 |
| | | | *Ruminococcaceae* | - | | Control | 23.70 | ± | 7.06 | | | | |
| | | | | | | Pic | 27.30 | ± | 2.77 | −1.606 | 3 | 0.207 | 0.068 |
| | | | | | | Res | 25.39 | ± | 5.64 | −2.062 | 3 | 0.131 | 0.068 |
| | Unnamed | Unnamed | *Unnamed* | - | | Control | 0.01 | ± | 0.00 | | | | |
| | | | | | | Pic | 0.00 | ± | 0.00 | 4.303 | 3 | 0.023 | 0.068 |
| **Phylum** | **Class** | **Order** | **Family** | **Genus** | **Species** | **Stilbenoid** | **Mean(%) ± SD** | | | | **df** | **P < 0.05** | **P < 0.075** |
| Actinobacteria | Coriobacteriia | Coriobacteriales | *Coriobacteriaceae* | *Gen.* | *sp.* | Control | 0.42 | ± | 0.33 | | | | |
| | | | | | | Pic | 0.48 | ± | 0.39 | −1.746 | 3 | 0.179 | 0.068 |
| | | | | | | Res | 0.48 | ± | 0.39 | −1.821 | 3 | 0.166 | 0.068 |
| | | | | *Adlercreutzia* | *sp.* | Control | 0.13 | ± | 0.09 | | | | |
| | | | | | | Pic | 0.09 | ± | 0.07 | 1.554 | 3 | 0.218 | 0.068 |
| | | | | *Collinsella* | *sp.* | Control | 0.14 | ± | 0.07 | | | | |
| | | | | | | Thu | 0.09 | ± | 0.03 | 1.194 | 3 | 0.148 | 0.068 |
| | | | | | *aerofaciens* | Control | 2.58 | ± | 0.55 | | | | |
| | | | | | | Pic | 2.43 | ± | 0.66 | 2.391 | 3 | 0.097 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | 0.13 | ± | 0.05 | | | | |
| | | | | | | Pic | 0.09 | ± | 0.05 | 4.546 | 3 | 0.020 | 0.068 |
| | | | | | | Thu | 0.07 | ± | 0.05 | 2.061 | 3 | 0.131 | 0.068 |

**Table A3.** *Cont.*

| | | | | | | | | | | | | Paired-T | Wilcoxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phylum** | **Class** | **Order** | **Family** | | | **Stilbenoid** | **Mean(%) ± SD** | | | **t** | **df** | **P < 0.05** | **P < 0.075** |
| Bacteroidetes | Bacteroidia | Bacteroidales | *Bacteroidaceae* | *Bacteroides* | *sp.* | Control | 0.06 | ± | 0.06 | | | | |
| | | | | | | Bat | 0.10 | ± | 0.07 | −26.712 | 2 | 0.001 | 0.109 |
| Firmicutes | Bacilli | Lactobacillales | *Aerococcaceae* | *Gen.* | *sp.* | Control | 0.03 | ± | 0.06 | | | | |
| | | | | | | Res | 0.02 | ± | 0.03 | 1.430 | 3 | 0.248 | 0.068 |
| | Clostridia | Clostridiales | *[Mogibacteriaceae]* | *Gen.* | *sp.* | Control | 0.40 | ± | 0.30 | | | | |
| | | | | | | Res | 0.49 | ± | 0.35 | −2.088 | 3 | 0.128 | 0.068 |
| | | | *Clostridiaceae* | *Clostridium* | *sp.* | Control | 0.07 | ± | 0.05 | | | | |
| | | | | | | Pic | 0.05 | ± | 0.05 | 2.378 | 3 | 0.098 | 0.068 |
| | | | | | | Res | 0.01 | ± | 0.01 | 1.808 | 3 | 0.168 | 0.068 |
| | | | | | | Thu | 0.04 | ± | 0.04 | 3.390 | 3 | 0.043 | 0.068 |
| | | | | *SMB53* | *sp.* | Control | 0.04 | ± | 0.01 | | | | |
| | | | | | | Pic | 0.02 | ± | 0.02 | 2.222 | 3 | 0.113 | 0.068 |
| | | | | | | Thu | 0.02 | ± | 0.02 | 2.008 | 3 | 0.138 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | 0.28 | ± | 0.05 | | | | |
| | | | | | | Pic | 0.19 | ± | 0.08 | 2.926 | 3 | 0.061 | 0.068 |
| | | | *Lachnospiraceae* | *[Ruminococcus]* | *sp.* | Control | 0.12 | ± | 0.04 | | | | |
| | | | | | | Bat | 0.09 | ± | 0.05 | 16.420 | 2 | 0.004 | 0.109 |
| | | | | | | Pic | 0.08 | ± | 0.05 | 4.482 | 3 | 0.021 | 0.068 |
| | | | | | | Thu | 0.06 | ± | 0.04 | 2.193 | 3 | 0.116 | 0.068 |
| | | | | | *sp.* | Control | 2.17 | ± | 1.71 | | | | |
| | | | | | | Res | 1.80 | ± | 1.39 | 2.251 | 3 | 0.110 | 0.068 |
| | | | | | *gnavus* | Control | 0.76 | ± | 0.34 | | | | |
| | | | | | | Thu | 0.91 | ± | 0.33 | -3.012 | 3 | 0.057 | 0.068 |
| | | | | *Anaerostipes* | *sp.* | Control | 0.05 | ± | 0.07 | | | | |
| | | | | | | Pic | 0.04 | ± | 0.07 | 2.485 | 3 | 0.089 | 0.068 |
| | | | | | | Res | 0.09 | ± | 0.09 | −2.516 | 3 | 0.086 | 0.068 |
| | | | | *Blautia* | *obeum* | Control | 0.43 | ± | 0.52 | | | | |
| | | | | | | Thu | 0.55 | ± | 0.59 | −2.929 | 3 | 0.061 | 0.068 |
| | | | | | *producta* | Control | 0.01 | ± | 0.01 | | | | |
| | | | | | | Res | 0.00 | ± | 0.00 | 2.185 | 3 | 0.117 | 0.068 |

**Table A3.** *Cont.*

| | | | | | | | | | | | | Paired-T | Wilcoxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phylum** | **Class** | **Order** | **Family** | | | **Stilbenoid** | **Mean(%) ± SD** | | | **t** | **df** | **P < 0.05** | **P < 0.075** |
| | | | | *Coprococcus* | *sp.* | Control | 1.88 | ± | 0.54 | | | | |
| | | | | | | Res | 1.62 | ± | 0.52 | 3.895 | 3 | 0.030 | 0.068 |
| | | | | *Dorea* | *sp.* | Control | 0.07 | ± | 0.04 | | | | |
| | | | | | | Res | 0.07 | ± | 0.04 | -4.817 | 2 | 0.040 | 0.715 |
| | | | | | *formicigenerans* | Control | 0.36 | ± | 0.16 | | | | |
| | | | | | | Thu | 0.49 | ± | 0.28 | −2.143 | 3 | 0.121 | 0.068 |
| | | | | *Lachnospira* | *sp.* | Control | 0.29 | ± | 0.44 | | | | |
| | | | | | | Pic | 0.33 | ± | 0.48 | −1.723 | 3 | 0.183 | 0.068 |
| | | | | *Roseburia* | *sp.* | Control | 0.19 | ± | 0.11 | | | | |
| | | | | | | Pic | 0.23 | ± | 0.09 | −1.555 | 3 | 0.218 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | 12.58 | ± | 0.44 | | | | |
| | | | | | | Res | 11.59 | ± | 0.80 | 3.468 | 3 | 0.040 | 0.068 |
| | | | *Ruminococcaceae* | *Faecalibacterium* | *prausnitzii* | Control | 2.01 | ± | 1.01 | | | | |
| | | | | | | Res | 2.79 | ± | 1.53 | −2.912 | 3 | 0.062 | 0.068 |
| | | | *[Mogibacteriaceae]* | *Gen.* | *sp.* | Control | 0.02 | ± | 0.03 | | | | |
| | | | | | | Res | 0.49 | ± | 0.35 | −2.088 | 3 | 0.128 | 0.068 |
| | | | | | | Thu | 0.01 | ± | 0.02 | 1.431 | 3 | 0.248 | 0.068 |
| | Unnamed | Unnamed | *Unnamed* | *Gen.* | *sp.* | Control | 0.01 | ± | 0.00 | | | | |
| | | | | | | Pic | 0.00 | ± | 0.00 | 4.303 | 3 | 0.023 | 0.068 |
| Proteobacteria | Gammaproteobacteria | Enterobacteriales | *Enterobacteriaceae* | *Gen.* | *sp.* | Control | 0.01 | ± | 0.01 | | | | |
| | | | | | | Thu | 0.00 | ± | 0.00 | 1.884 | 3 | 0.156 | 0.068 |

**Table A4.** Identified taxa based on Comparison 2, which used as a baseline the magnitude of change (growth or decline) in relative abundance between Control 0 h and Control 24 h, and compared that to the magnitude of change between Control 0 h and each of the 6 stilbenoid fermentations. Taxa displayed Paired-T $p$ value <0.05 and/or Wilcoxon Signed Rank $p$ value <0.075. Bolded values are those within these ranges. Gen. = unnamed genus, sp. = unnamed species.

| | | | | | | | | | | | | **Paired-T** | **Wilcoxon** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phylum** | **Class** | **Order** | **Family** | | | **Stilbenoid** | **Mean(%) ± SD** | | | **t** | **df** | **P < 0.05** | **P < 0.075** |
| Actinobacteria | Coriobacteriia | Coriobacteriales | *Coriobacteriaceae* | - | | Control | 13.01 | ± | 51.37 | | | | |
| | | | | | | Pic | 6.73 | ± | 52.38 | 2.465 | 3 | 0.090 | 0.068 |
| Firmicutes | Clostridia | Clostridiales | *Lachnospiraceae* | - | | Control | −20.13 | ± | 9.71 | | | | |
| | | | | | | Res | −22.88 | ± | 10.24 | 4.197 | 3 | 0.025 | 0.068 |
| | | | *Ruminococcaceae* | - | | Control | 4.68 | ± | 35.06 | | | | |
| | | | | | | Pic | 21.94 | ± | 27.47 | −1.604 | 3 | 0.207 | 0.068 |
| | | | | | | Res | 12.17 | ± | 28.65 | −1.894 | 3 | 0.155 | 0.068 |
| **Phylum** | **Class** | **Order** | **Family** | **Genus** | **Species** | **Stilbenoid** | **Mean(%) ± SD** | | | | **df** | **P < 0.05** | **P < 0.075** |
| Actinobacteria | Coriobacteria | Coriobacteriales | *Coriobacteriaceae* | *Gen.* | *sp.* | Control | 43.71 | ± | 85.20 | | | | |
| | | | | | | Pic | 59.03 | ± | 95.62 | -2.563 | 3 | 0.083 | 0.068 |
| | | | | | | Res | 58.79 | ± | 92.21 | -2.608 | 3 | 0.080 | 0.068 |
| | | | | *Collinsella* | *Sp.* | Control | 24.32 | ± | 63.97 | | | | |
| | | | | | | Thu | −21.06 | ± | 13.23 | 1.722 | 3 | 0.183 | 0.068 |
| | | | | | *aerofaciens* | Control | −1.03 | ± | 51.47 | | | | |
| | | | | | | Pic | −6.22 | ± | 51.19 | 2.685 | 3 | 0.075 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | 27.75 | ± | 80.59 | | | | |
| | | | | | | Oxy | −0.93 | ± | 94.16 | 6.272 | 2 | 0.024 | 0.109 |
| | | | | | | Pic | −3.70 | ± | 90.64 | 3.261 | 3 | 0.047 | 0.068 |
| | | | | | | Thu | −39.16 | ± | 10.03 | 1.726 | 3 | 0.183 | 0.068 |

**Table A4.** *Cont.*

| | | | | | | | | | | | Paired-T | Wilcoxon |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phylum** | **Class** | **Order** | **Family** | | | **Stilbenoid** | **Mean(%) ± SD** | | | **t** | **df** | **P < 0.05** | **P < 0.075** |
| Firmicutes | Clostridia | Clostridiales | *[Mogibacteriaceae]* | *Gen.* | *sp.* | Control | 72.05 | ± | 96.46 | | | | |
| | | | | | | Res | 121.96 | ± | 121.92 | −2.783 | 3 | 0.069 | 0.068 |
| | | | *Clostridiaceae* | *Clostridium* | *sp.* | Control | −54.19 | ± | 28.78 | | | | |
| | | | | | | Pic | −62.90 | ± | 27.96 | 3.960 | 3 | 0.029 | 0.068 |
| | | | | | | Res | −90.28 | ± | 15.89 | 1.908 | 3 | 0.152 | 0.068 |
| | | | | | | Thu | −79.31 | ± | 22.65 | 3.901 | 3 | 0.030 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | 122.65 | ± | 206.83 | | | | |
| | | | | | | Pic | 6.93 | ± | 40.25 | 1.353 | 3 | 0.269 | 0.068 |
| | | | *Lachnospiraceae* | *[Ruminococcus]* | *sp.* | Control | 32.18 | ± | 68.47 | | | | |
| | | | | | | Bat | −3.23 | ± | 69.11 | 4.448 | 2 | 0.047 | 0.109 |
| | | | | | | Pic | −7.02 | ± | 69.37 | 8.253 | 3 | 0.004 | 0.068 |
| | | | | | | Thu | −41.13 | ± | 50.91 | 1.953 | 3 | 0.146 | 0.068 |
| | | | | | *sp.* | Control | 15.46 | ± | 20.76 | | | | |
| | | | | | | Res | −3.29 | ± | 12.72 | 3.947 | 3 | 0.029 | 0.068 |
| | | | | | *gnavus* | Control | −12.89 | ± | 30.72 | | | | |
| | | | | | | Thu | 8.24 | ± | 40.57 | −2.244 | 3 | 0.111 | 0.068 |
| | | | | *Blautia* | *obeum* | Control | −29.83 | ± | 35.61 | | | | |
| | | | | | | Thu | −5.56 | ± | 32.11 | −3.763 | 3 | 0.033 | 0.068 |
| | | | | *Coprococcus* | *sp.* | Control | −5.31 | ± | 11.92 | | | | |
| | | | | | | Res | −19.86 | ± | 3.60 | 2.883 | 3 | 0.063 | 0.068 |
| | | | | *Dorea* | *sp.* | Control | 16.18 | ± | 75.27 | | | | |
| | | | | | | Oxy | 59.34 | ± | 95.43 | −9.591 | 2 | 0.011 | 0.109 |
| | | | | | *formicigenerans* | Control | −5.41 | ± | 27.27 | | | | |
| | | | | | | Thu | 30.22 | ± | 52.23 | −2.397 | 3 | 0.096 | 0.068 |
| | | | | *Lachnospira* | *sp.* | Control | 69.70 | ± | 26.12 | | | | |
| | | | | | | Pic | 128.21 | ± | 95.39 | −1.274 | 3 | 0.292 | 0.068 |
| | | | | *Roseburia* | *sp.* | Control | −63.94 | ± | 38.85 | | | | |
| | | | | | | Pic | −56.62 | ± | 37.62 | −1.597 | 3 | 0.209 | 0.068 |
| | | | | *Gen.* | *sp.* | Control | −19.04 | ± | 18.36 | | | | |
| | | | | | | Res | −25.50 | ± | 16.67 | 3.433 | 3 | 0.041 | 0.068 |
| | | | *Ruminococcaceae* | *Faecalibacterium* | *prausnitzii* | Control | −0.51 | ± | 62.49 | | | | |
| | | | | | | Res | 36.58 | ± | 87.95 | −2.806 | 3 | 0.068 | 0.068 |

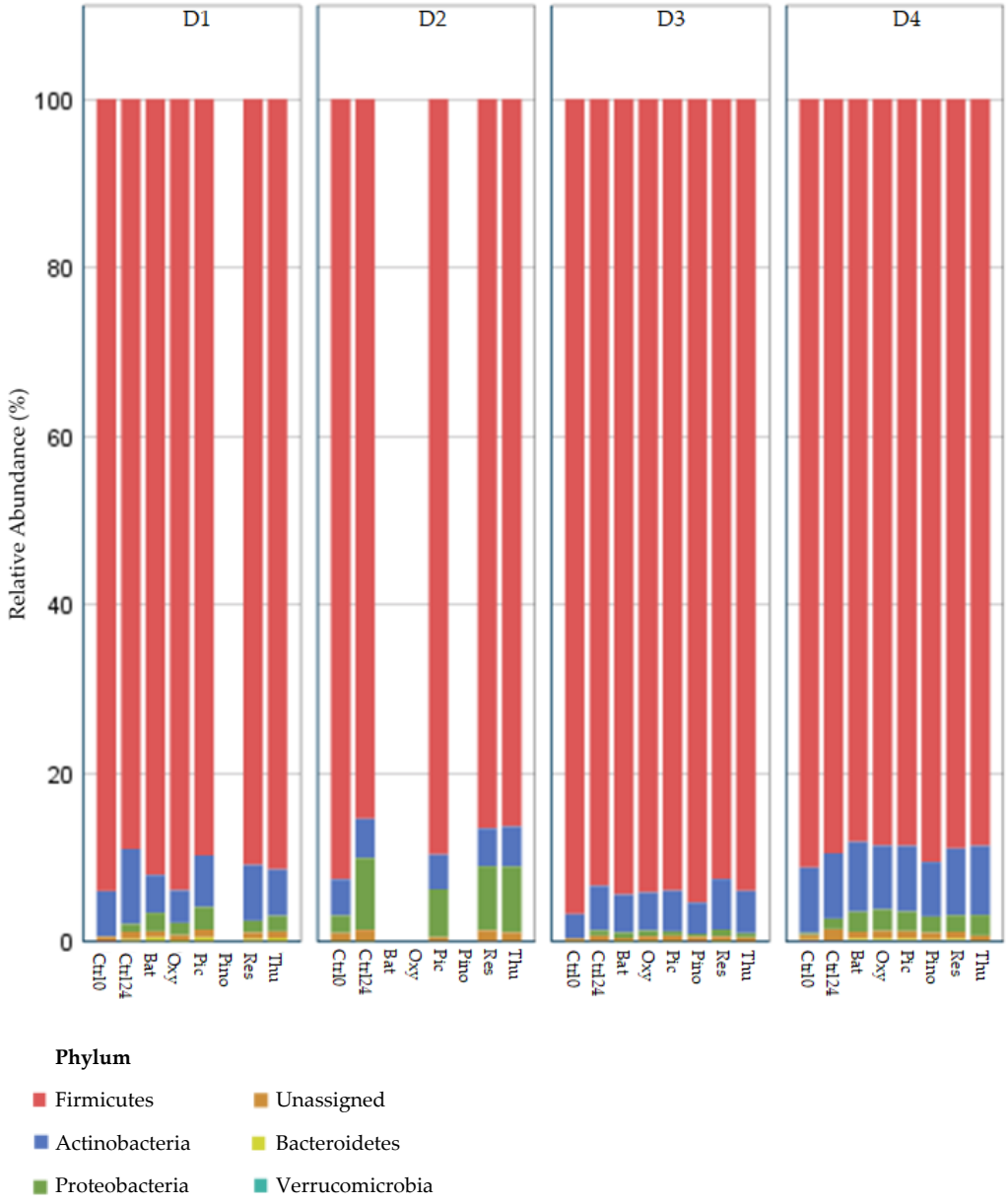


**Figure A1.** Bacterial composition at the phylum level, per donor. D# denotes the donor; Ctrl0, control at 0 h; Ctrl24, control at 24 h; Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene (all stilbenoids at 24 h).

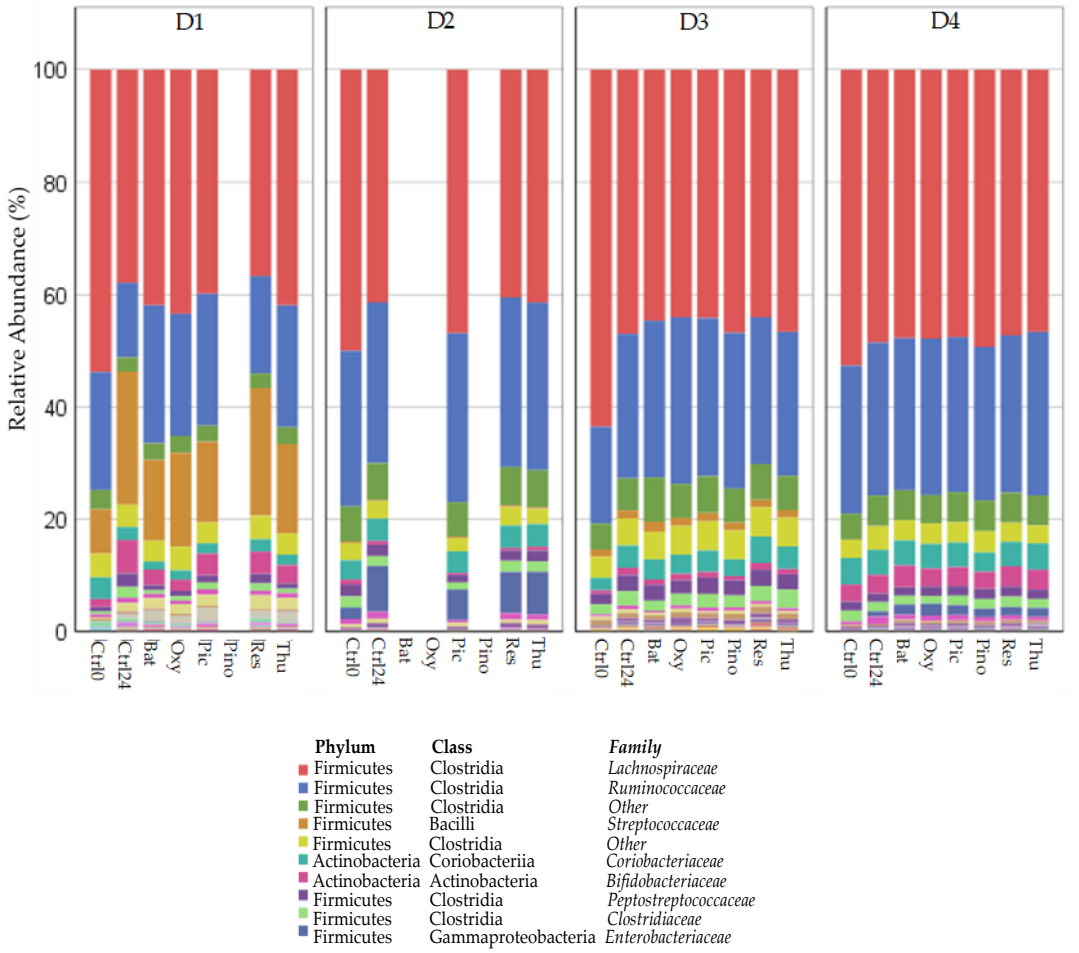


**Figure A2.** Bacterial composition at the family level, per donor, for the 10 most abundant taxa. D# denotes the donor; Ctrl0, control at 0 h; Ctrl24, control at 24 h; Bat, batatasin III; Oxy, oxyresveratrol; Pic, piceatannol; Pino, pinostilbene; Res, trans-resveratrol; Thu, thunalbene (all stilbenoids at 24 h).

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**Sample Availability:** Samples of the compounds are not available from the authors.

molecules

MDPI

Article

# Metabolism of Stilbenoids by Human Faecal Microbiota

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**Abstract:** Stilbenoids are dietary phenolics with notable biological effects on humans. Epidemiological, clinical, and nutritional studies from recent years have confirmed the significant biological effects of stilbenoids, such as oxidative stress protection and the prevention of degenerative diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases. Stilbenoids are intensively metabolically transformed by colon microbiota, and their corresponding metabolites might show different or stronger biological activity than their parent molecules. The aim of the present study was to determine the metabolism of six stilbenoids (resveratrol, oxyresveratrol, piceatannol, thunalbene, batatasin III, and pinostilbene), mediated by colon microbiota. Stilbenoids were fermented in an in vitro faecal fermentation system using fresh faeces from five different donors as an inoculum. The samples of metabolized stilbenoids were collected at 0, 2, 4, 8, 24, and 48 h. Significant differences in the microbial transformation among stilbene derivatives were observed by liquid chromatography mass spectrometry (LC/MS). Four stilbenoids (resveratrol, oxyresveratrol, piceatannol and thunalbene) were metabolically transformed by double bond reduction, dihydroxylation, and demethylation, while batatasin III and pinostilbene were stable under conditions simulating the colon environment. Strong inter-individual differences in speed, intensity, and pathways of metabolism were observed among the faecal samples obtained from the donors.

**Keywords:** bacteria colon model; fecal fermentation; metabolites; phenolics; polyphenols; stilbenoids; liquid chromatography high resolution mass spectrometry

## 1. Introduction

Stilbenoids are dietary phenolics that occur in a wide range of edible fruits and seeds, such as grapes (*Vitis vinifera*), peanuts (*Arachis hypogaea*), sorghum (*Sorghum bicolor*), and some tree species (*Pinus* spp. and *Picea* spp.) [1]. Resveratrol (3,4′,5-trihydroxystilbene) is the most studied stilbenoid, and is commonly associated with the French paradox, where resveratrol is thought to lower the incidence of coronary heart disease, despite a high intake of saturated fat in the French population [2]. In mice studies, both in vitro and in vivo, resveratrol has shown strong anti-inflammatory activity by a reduction of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), an increase of interleukin 10 (IL-10), and a reduced expression of prostaglandin E

synthase-1 (PGES-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [3,4]. Therefore, stilbenoid could act as an anti-inflammatory agent through some of the same mechanisms as nonsteroidal antiphlogistic drugs. Resveratrol has also been reported to affect various factors and metabolic targets associated with oxidative stress [1,5,6]. Among these, resveratrol has a strong affinity to quinone reductase 2, with a dissociation constant as low as 35 nM, making it the strongest known inhibitor so far, which, in turn, may regulate the expression of cellular antioxidant enzymes and cellular resistance to oxidative stress [7]. Resveratrol further interacts with a large number of receptors and enzymes that could plausibly make major contributions to its biological effects. Both in vitro and in vivo, resveratrol treatment upregulates mammalian target of rapamycin (mTOR), sirtuin 1 (SIRT1), and adenosine monophosphate-activated protein kinase (AMPK), which influence the regulation of metabolism in multiple tissues [8,9]. However, the in vivo importance is rather more relevant to insects than to mammals. Structural analogs of resveratrol are present in medicinal plants and show significant bioactivity. For instance, piceatannol (3,3′,4′,5-tetrahydroxystilbene) is found in plants such as grapes (*V. vinifera*), passion fruit (*Passiflora edulis*), Japanese knotweed (*Polygonum cuspidatum*), and Norway spruce (*P. abies*) [10]. Compared to resveratrol, piceatannol shows greater biological activity as an inhibitor of COX-2 and of the constitutive photomorphogenesis 9 signalosome (CSN)-associated kinase [11], possibly due to its better solubility in $H_2O$. Moreover, piceatannol inhibits the activation of p40 and p56 protein tyrosine kinases and NF-κB [12]. Another analog of resveratrol, pinostilbene (3,4′-dihydroxy-5-methoxystilbene), found in Siberian pine (*P. sibirica*), showed an inhibition of dopamine-induced cell death through extracellular signal–regulated kinase (ERK 1/2) activation in an in vitro study, was shown to alleviate the loss of motor function seen on aging in vivo [13]. Oxyresveratrol (2′,3,4′,5-tetrahydroxystilbene), found in white mulberry (*Morus alba*), exhibited, among other effects, the inhibition of TNF-α production and stronger antioxidant activity than resveratrol [14]. Many of these molecules have been subjected to clinical trials and are being investigated as clinical drugs [15–17].

Phenolics are intensively metabolically transformed in intestinal epithelial cells and transported through the basolateral membrane in the form of conjugates [18]. Some typical intestinal epithelium metabolites include *O*-β-glucuronides, 3-*O*-sulfates, or their methoxy-derivatives. However, a large portion of the phenolic compounds escape intestinal absorption and undergo their microbial metabolic conversion in the colon [19]. Colon catabolism is an important phase of the pharmacokinetics of chemical entities in the human body. Its knowledge is an important prerequisite for bioassay validation or for the development of more active substituents. Colonic catabolites might be more biologically relevant forms of the compounds, and their use in bioassays is a more realistic reflection of the compound's bioactivity. To date, only resveratrol and its fate in the human colon has been investigated, finding three metabolites: dihydroresveratrol, 3,4′-dihydroxy-trans-stilbene, and 3,4′-dihydroxybibenzyl (lunularin) [20]. The bioactivity of dihydroresveratrol, found by in vivo and in vitro studies, includes antioxidant [21] and anti-inflammatory [22] activity.

Thus, the aim of the present study was to investigate whether six selected stilbenoids (batatasin III, oxyresveratrol, piceatannol, pinostilbene, resveratrol, thunalbene) undergo metabolic transformation by human colon microbiota and thereafter detect their main metabolites by liquid chromatography mass spectrometry (LC/MS).

## 2. Results

The in vitro faecal fermentation system, using fresh faeces from five different donors (D) as inoculum, was performed to analyze the metabolism of selected stilbenoids (batatasin III, oxyresveratrol, piceatannol, pinostilbene, resveratrol, thunalbene) in the human colon. As seen in Figure 1, significant differences in the microbial transformation among stilbene derivatives were observed. Four stilbenoids (resveratrol, oxyresveratrol, piceatannol and thunalbene) were metabolically transformed to new products, while batatasin III and pinostilbene were stable in the colon environment.

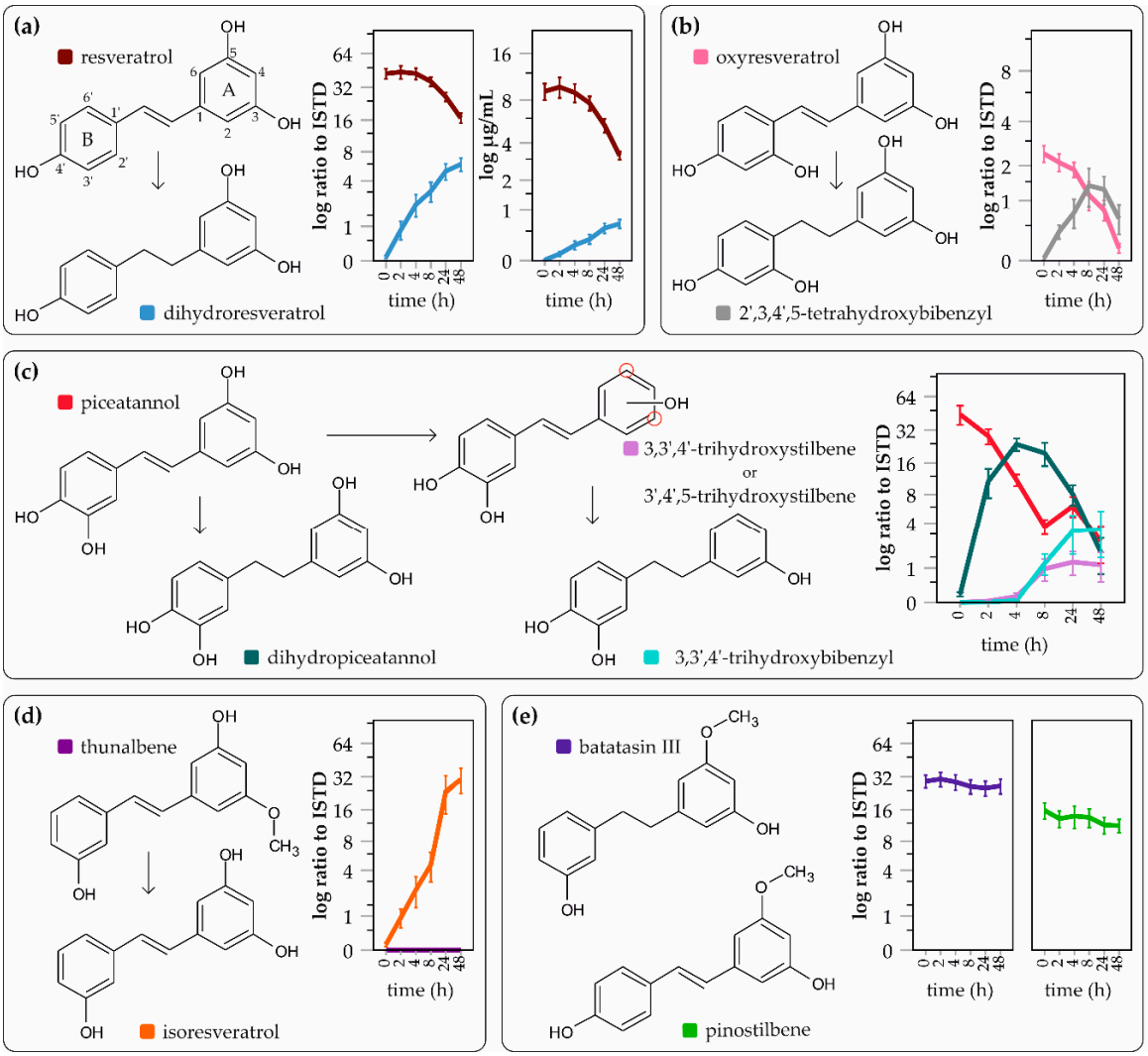


**Figure 1.** The effect of bacterial metabolism on (**a**) resveratrol ($N$ = 5); (**b**) oxyresveratrol ($N$ = 3); (**c**) piceatannol ($N$ = 5); (**d**) thunalbene ($N$ = 5); (**e**) batatasin III ($N$ = 4); and pinostilbene ($N$ = 5); values obtained from LC/MS are expressed as ratios of produced metabolite to internal standard (ISTD) as means ± 1 SE ($p < 0.05$); N represents the number of donors analyzed.

The only metabolite of resveratrol detected in our model was dihydroresveratrol, which was not further metabolized and remained stable in the colon environment. Amounts of resveratrol and dihydroresveratrol were monitored: after 48 h, the concentration of resveratrol decreased to 3.2 ± 0.9 µg/mL (mean ± SD) from the initial 9.1 ± 4.4 µg/mL. The concentration of dihydroresveratrol rose gradually to a final concentration of 0.7 ± 0.4 µg/mL after 48 h of fermentation. The metabolism of oxyresveratrol was similar to resveratrol, and one metabolite, 2′,3,4′,5-tetrahydroxybibenzyl, formed by double bond reduction, was detected. It reached its maximum level after 24 h and then was further degraded to still unknown products. The metabolism of piceatannol was more complex. The main metabolic pathway was the double bond reduction of the connective chain, forming dihydropiceatannol, which reached its maximum level after 4 h and then was also further degraded to still unknown products. In some donors, piceatannol was dehydroxylated at one of the meta positions on ring A, forming trihydroxystilbene different than resveratrol or isoresveratrol (see Appendix A, Figure A1), and was further metabolized to 3,3′,4′-trihydroxybibenzyl. This reaction was much slower than the formation of dihydropiceatannol and occurred between 4 to 8 h of fermentation. Even though thunalbene was not observed in the samples, its demethylated

metabolite, isoresveratrol (3,3′,5-trihydroxystilbene), was detected. This metabolite was stable and was not further metabolized or degraded. Batatasin III and pinostilbene did not form any metabolites and were found to be stable in the colon environment.

Strong inter-individual differences were observed among the donors, as seen in Appendix B Figure A2. Resveratrol was gradually metabolized in all samples to dihydroresveratrol, but the final concentrations varied among the donors from 77 ± 1% (mean ± SD; D2) to 11 ± 1% (D5). Oxyresveratrol was metabolized in each sample at different intensities, 100 ± 0% (D1), 84 ± 1% (D3), and 98 ± 2% (D5). Piceatannol, after 48 h of fermentation, was more than 99.6% metabolized in four of the samples, except for sample D2, where 12 ± 1% of piceatannol was still present at the end of the fermentation. Piceatannol was metabolized to dihydropiceatannol in all faecal samples, but in two out of the five cases (D4 and D5) it was metabolized to 3,3′,4′-trihydroxystilbene or 3′,4′,5-trihydroxystilbene and further to 3,3′,4′-trihydroxybibenzyl. Similarly, thunalbene was metabolized to isoresveratrol in only three out of the five faecal samples (D2, 3 and 4), while in the others no metabolites were detected.

## 3. Discussion

The current study provides new information about the biotransformation of six stilbenoids by human gut microbiota, depending on their structural molecular properties. The obtained data show that stilbenoids differ in their stability in a colonic environment. Whereas batatasin III and pinostilbene did not produce any metabolites, resveratrol, oxyresveratrol, piceatannol, and thunalbene were intensively metabolized by colon microbiota. Three main reactions were found to be ongoing in our human colon model: double bond reduction, dihydroxylation, and demethylation. An important factor in the course of the reactions was the location of the hydroxyl and methyl groups. Differences in the intensity, rate, and spectrum of metabolites were also observed among the faecal samples obtained from different donors.

The only resveratrol metabolite detected in this study was dihydroresveratrol, which is in agreement with other in vitro and in vivo studies [23–25]. Another study, using a similar model, described two more metabolites, 3,4′-dihydroxy-*trans*-stilbene and 3,4′-dihydroxybibenzyl (lunularin), which were detected in six out of seven faecal samples [20]. Their absence in our study might be caused by a different composition of bacterial species in faecal samples or different initial concentrations of stilbenoids, which might saturate some enzymatic catabolic pathways and change the method of metabolite formation. It is evident that the course of the catabolic reaction was affected by the inter-individual differences in the bacterial composition of the faecal samples. In our study, resveratrol had been gradually catabolized, and its final concentration, after 48 h of fermentation, ranged from 77 ± 1% (D2) to 11 ± 1% (D5). This gradual decrease contrasts with another study [20] that reported a complete degradation of resveratrol in a time frame of 2 to 24 h. This might be partly caused by different initial concentrations of resveratrol (80 µM vs. 44 µM in our study), lower inoculum, medium composition, or simply differences in the donor's microbial composition. Previously, we reported bacterial composition in a subset of samples reported here (time points 0 and 24 h; and donors D1-D4), showing major differences between this study [26] and the study from [20]. While Faecalibacterium (12–21% of DNA) and Bacteroides (9–16% of DNA) were the most abundant group in [20], our fermentations were dominated by Clostridia at time points 0 h and 24 h [26]. Mean *Faecalibacteium prausnitzii* abundance at time point 0 h was only 2.01 ± 1.01% and Bacteroides were only 0.06 ± 0.06%. In a previous study, 43 bacteria, mostly gut-associated strains, were screened for their capacity to catabolize resveratrol [23], from which 11 strains were capable of metabolizing resveratrol by more than 20%, among them *Escherichia coli* ATCC 25922, *Bacillus cereus* NCTR-466, and *Achromobacter denitrificans* NCTR-774 had transformed resveratrol almost completely within 24 h.

Similar to resveratrol and oxyresveratrol, piceatannol was metabolized to dihydropiceatannol by colon microbiota via hydrogenation of the ethylene bridge. However, another pathway has also been detected. Faecal bacteria from some donors were able to cleave the hydroxyl group on ring A in one of

the meta positions and form trihydroxystilbene, different from resveratrol or isoresveratrol, which was further dehydroxylated to 3,3′,4′-trihydroxybibenzyl.

In the present study, three derivatives of stilbenoids formed by double bond reduction were detected. Their further fate in the colon model was dependent on the position of the hydroxyl group. Dihydroresveratrol, with only one hydroxyl group on ring B in para position, was not further metabolized or degraded, and it was the end-product of resveratrol colon metabolism. The catabolite of oxyresveratrol, 2,3,3′,5′-tetrahydroxystilbene, has two hydroxyl groups in para and ortho positions on ring B. Concentration change of this metabolite was more dynamic in this model, reaching its maximum concentration at 24 h and then further degrading to still unknown products. The least stable of these metabolites was dihydropiceatannol, which had two hydroxyl groups in para and meta position on ring B. It has been shown that dihydroresveratrol could be produced as an end-product or transient intermediate, depending on the donor [20], so the high persistence of dihydroresveratrol in the present study might only be an observed effect particular to our donors. The marked influence of colon microbiota composition on the metabolism of polyphenols has also been well reported by other authors [19].

Thunalbene was not observed as a parent compound in the samples, possibly because of binding to the matrix or polymerization. However, its demethylated catabolite, isoresveratrol, was detected in three out of the five donors (D2, 3, and 4). Interestingly, batatasin III and pinostilbene, other methylated stilbenes, were demonstrably stable in the colon model. This matches results obtained in a study of pterostilbene (3,5-dimethoxy-4′-hydroxystilbene) metabolism in the colons of mice, where pinostilbene was found as its main product; however, no metabolites of pterostilbene with a reduced double bond were identified [18,27]. This result indicates that the position of the methoxy group could play an important role in its demethylation, as well as in the reduction of the ethylene bridge by intestinal microbiota.

In our previous study [26] the microbiota composition of donors D1-4 had been observed. Compared to the others, donors D1 and D2 seemed to be very atypical, with a higher representation of class Bacilli (*Streptococcaceae* family) in donor D1 and a higher representation of the *Enterobacteriaceae* family in donor D2. Due to the lack of the compounds, the full set of all six stilbenoids was fermented only by samples from donors D1, D3, and D5. Microbiota from the faecal sample of donor D1 was able to metabolize about half of the resveratrol and completely metabolize oxyresveratrol and piceatannol within 48 h, but it was not metabolizing thunalbene. Microbiota from donor D3 were effectively metabolizing resveratrol (20 $\pm$ 1% occurred after 48 h), and demethylating thunalbene to isoresveratrol, but was the least effective in metabolizing oxyresveratrol (84 $\pm$ 1% occurred after 48 h). Microbiota from donor D5 were the most effective in metabolizing resveratrol (11 $\pm$ 1% occurred after 48 h), and were able to dehydroxylate piceatannol. However, similar to donor D1, these microbiota were not able to metabolize thunalbene to isoresveratrol. Microbiota from donor D2 were the least effective in metabolizing resveratrol (77 $\pm$ 1% occurred after 48 h), and piceatannol (12 $\pm$ 1% occurred after 48 h) but seem to be the most effective in metabolizing thunalbene. Microbiota of donor D4 were not very effective in metabolizing either resveratrol or thunalbene but were as efficient as an inoculum of donor D5 by dehydroxylating piceatannol.

In conclusion, it has been shown that some stilbenoids are intensively catabolized by the colon microbiota, whereas others seem to be stable in the colon environment. In our model, the degree of substitution played an important role on the level of molecular fragility. Derivatives with the hydroxy group in the para position were less fragile than the others, and further study of their behavior in the colon is needed. The rate, intensity, and the pathways of metabolism are closely associated with colon microbiota composition. However, the role of particular bacterial species on the metabolism of stilbenoids is not clear, and thus future research should also be focused on inter-individual differences and work with a larger number of donors. To our knowledge, this is the first study investigating the metabolic fate of stilbenoids other than resveratrol in the faecal human colon model. This study has

also implication on future screening assays for biological activities, so that relevant metabolites can be included.

## 4. Materials and Methods

### *4.1. In vitro Faecal Fermentation System*

A slightly modified fermentation model, previously described by other authors [28,29], was used to mimic the conditions in the human colon.

#### 4.1.1. Fermentation Medium

The fermentation medium was prepared as a solution of 225 mL distilled water, 1.12 g tryptone, 56.25 µL of micromineral solution (2.64 g $CaCl_2 \cdot 2H_2O$, 2 g $MnCl_2 \cdot 4H_2O$, 0.20 g $CoCl_2 \cdot 6H_2O$, 1.60 g $FeCl_3 \cdot 6H_2O$, and distilled water up to 20 mL), 112.5 mL of macromineral solution (7.14 g of $Na_2HPO_4 \cdot 2H_2O$, 6.20 g $KH_2PO_4$, 0.60 g $MgSO_4 \cdot 7H_2O$, and distilled water up to in 1 L), 112.5 mL of $CO_3$ buffer (1 g $NH_4HCO_3$, 8.75 g $NaHCO_3$, and distilled water up to 250 mL), and 562.5 µL of 0.1% resazurin solution. All chemicals were obtained from Merck (Darmstadt, Germany) and stored at 4 °C for up to 1 month. The prepared fermentation medium was covered with aluminum foil and stored at 4 °C until the next day.

#### 4.1.2. Sodium Phosphate Buffer and Reducing Solution

The sodium phosphate buffer for preparation of the faecal slurries was made of 1.77 g $KH_2PO_4$ in 195 mL distilled water and 3.62 g of $Na_2HPO_4$ in 305 mL distilled water (both 1/15 M). Afterward, the buffer's pH was modified to 7.0 by hydrochloric acid and stored at 4 °C for up to one month. The reducing solution was prepared just before the experiment from 125 mg cysteine hydrochloride, 0.8 mL 1M NaOH, 125 mg $Na_2S$, and distilled water up to 20 mL.

#### 4.1.3. Stilbenoid Preparation

Batatasin III, piceatannol, thunalbene, and pinostilbene were purchased from ChemFaces (China) at 98% purity; resveratrol, oxyresveratrol, and [$^{13}C_6$] *trans*-resveratrol were obtained from Merck (Darmstadt, Germany) at 98% purity. Stock solutions for fermentation experiments were prepared at a concentration of 10 mg/mL in DMSO (dimethylsulfoxide; Sigma-Aldrich, Prague, Czech Republic) and kept at 4 °C. Analytical standard stock solutions for LC/MS were prepared in methanol (1 mg/mL) and stored at −80 °C.

#### 4.1.4. Faecal Samples and Ethics Statement

Human faecal samples were collected in October and November 2016, at the Czech University of Life Sciences in Prague, Czech Republic from 5 healthy volunteers. These volunteers were aged 23 to 29 years, with a mean BMI of 24.6, no history of gastrointestinal disease and no antibiotic treatment for at least 3 months prior to the experiment. Female volunteers were neither pregnant nor lactating. All donors followed an omnivorous diet in their daily life and a two-day low polyphenol diet before the sample collection. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Czech University of Life Sciences in Prague (ZEK/22/09/2017). Samples were collected into 1 L plastic container, tied in a plastic bag with GENbag anaer (Biomérieux, Lyon, France) and kept at 37 °C for 2 h maximum. Fresh faeces were homogenized in a stomacher bag for 30 s with a sodium phosphate buffer and the obtained 32% faecal slurry was filtrated through a nylon mesh.

#### 4.1.5. In Vitro Incubations

The fermentation medium and sodium phosphate buffer were boiled with cotton cups and cooled to approximately 37 °C while they were purged with nitrogen gas free of oxygen (approximately 30 min). In the end, the color of the medium changed from blue to pink. The medium's pH was adjusted to pH 7.0 using HCl. The fermentation bottles (20 mL) were filled with 16.8 mL of medium and sealed with PTFE/aluminum caps. The reducing solution (0.8 µL) was added through the septa and, after full decolorization of resazurin, 20 µL of the tested compound and 2 mL of the faecal slurry were added. 20 µL of DMSO, instead of the tested compound, was added to the negative control vials, and 2 mL of the sodium phosphate buffer, instead of the faecal slurry, was added to the positive control vials. The incubation was carried out in a shaking water bath at 37 °C, at 100 strokes per minute. Samples (3 mL) were collected at 0, 2, 4, 8, 24, and 48 h with a syringe through the septa and stored at −80 °C until analysis.

### *4.2. LC/MS analyses*

#### 4.2.1. Standards

Standards were prepared as 1% Methanol/Formic Acid solution. Stock solutions were prepared at a concentration of 10 mg/mL in DMSO (Sigma-Aldrich, Stribrna Skalice, Czech Rep) and kept at 4 °C.

#### 4.2.2. Sample Purification

A liquid-liquid extraction was used for the purification of samples. The samples from fermentations were centrifuged (5 min; 15,000 rpm/min), and 400 µL of supernatant was diluted with 2 mL of ultra-pure water (Milipore, Bedford, MA, United States of America); 20 µL of [$^{13}C_6$] *trans*-resveratrol solution in methanol (2 µg/mL) was added as an internal standard. Then, the samples were extracted three times by 2.5 mL ethyl acetate (VWR Chemicals, Stribrna Skalice, Czech Republic). After purification, the combined organic phase was dried under nitrogen gas and re-dissolved in 1 mL of methanol (VWR Chemicals, Stribrna Skalice, Czech Republic) with 1% formic acid (Fisher Scientific, Merelbeke, Belgium). Final samples were analyzed by LC/MS.

#### 4.2.3. LC/MS Analysis of Metabolites

Analyses were performed on a LC/MS system consisting of an UHPLC chromatograph Ultimate 3000 Thermofisher Scientific (Sunnyvale, CA, USA) coupled with quadrupole time of flight (Q-TOF) mass spectrometer with ultra-high resolution and high mass accuracy (HRAM) Impact II (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source.

Chromatography was carried out on a Kinetex 1.7 µm F5 100 Å 100 × 2.1 mm column (Phenomenex, Torrance, CA, USA) using a mobile phase consisting of 0.1% formic acid (solvent A) and methanol (solvent B). The binary gradient was run as follows: 0–3 min isocratic at 20 % B, 3–6 min from 20 % to 50 % B, 6–15 min from 50% to 100 % B, and 15–20 min isocratic at 20 % B. The flow rate was kept at 0.2 mL/min, and the column oven was adjusted to 35 °C. The injected volume was 5 µL.

The ESI source was operated in the negative mode with parameters listed in Appendix C, Table A1. The identity of each detected compound was confirmed by MS/MS fragmentation spectra collected at three collision energy levels (20, 30 and 50 eV). Data acquisition was performed using HyStar 3.2 SR4, QTOF series 4.0 (Bruker Daltonics–Germany), and Chromeleon Xpress (Thermo Fisher Scientific) software, and the obtained data were processed by DataAnalysis 4.3. and TASQ 1.4 (both Bruker Daltonics–Germany). For calibration, commercially available standards of resveratrol, dihydroresveratrol, oxyresveratrol, piceatannol, thunalbene, batatasin III and pinostilbene were used each at 6 concentration levels in the range of 20–1000 ng/mL. As an internal standard, 20 µL of *trans*-[$^{13}C_6$] resveratrol at a concentration of 2 µg/mL was used.

### 4.2.4. Statistical Evaluation

Resveratrol, thunalbene, piceatannol and pinostilbene were used in five biological repetitions. Oxyresveratrol and batatasin III were used in three and four repetitions, respectively. All samples were measured by LC/MS in triplicates. Values are expressed as a mean ± standard error. Microsoft Excel and SPSS (IBM corp.) version 25 were used for basic statistical analysis and graph creation.

**Author Contributions:** Conceptualization J.H. and V.J.; methodology, J.H., P.M.; software, P.M., O.V.; validation, J.H., P.M.; formal analysis, J.H., J.D.J., P.K.; investigation, O.V., V.J., J.D.J., P.M.; resources, J.H., K.S.; data curation, P.M.; writing—original draft preparation, V.J., O.V.; writing—review and editing, J.H., P.M., J.D.J.; visualization, J.H., V.J.; supervision, J.H.; project administration, J.H., P.K.; funding acquisition, K.S., J.H., O.V.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## Appendix A

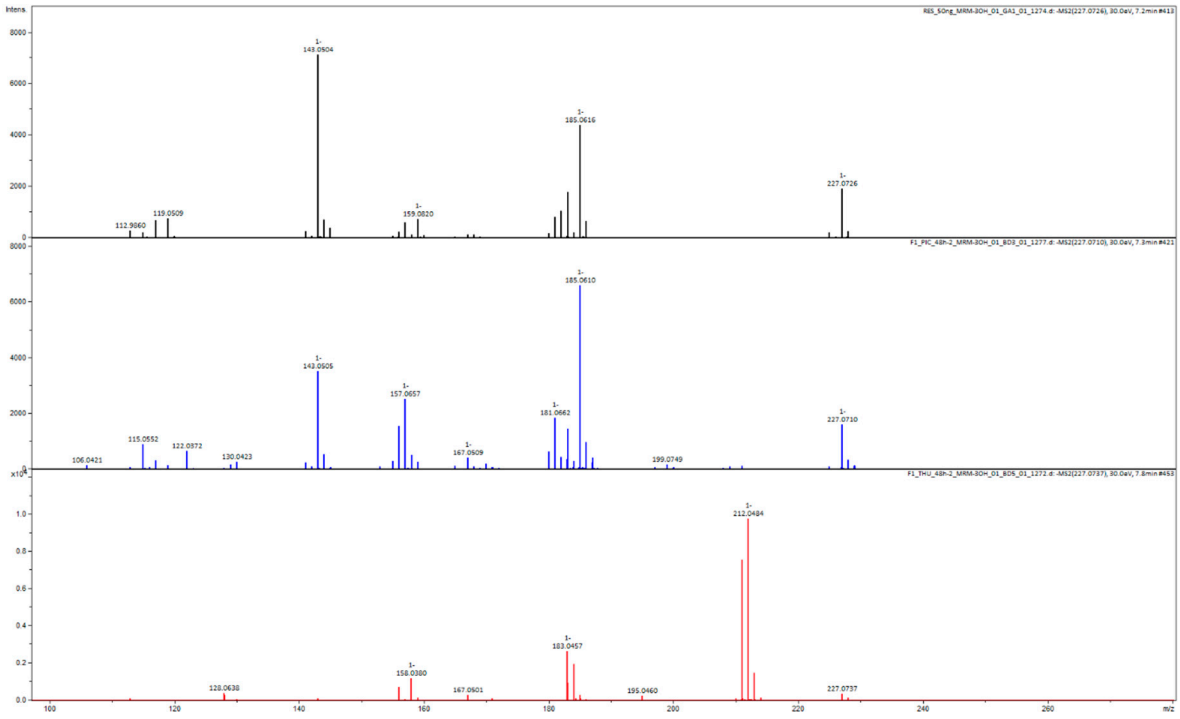


**Figure A1.** MS/MS spectra of resveratrol, 3,3′,4′-trihydroxystilbene/3′,4′,5-trihydroxystilbene and isoresveratrol.

## Appendix B

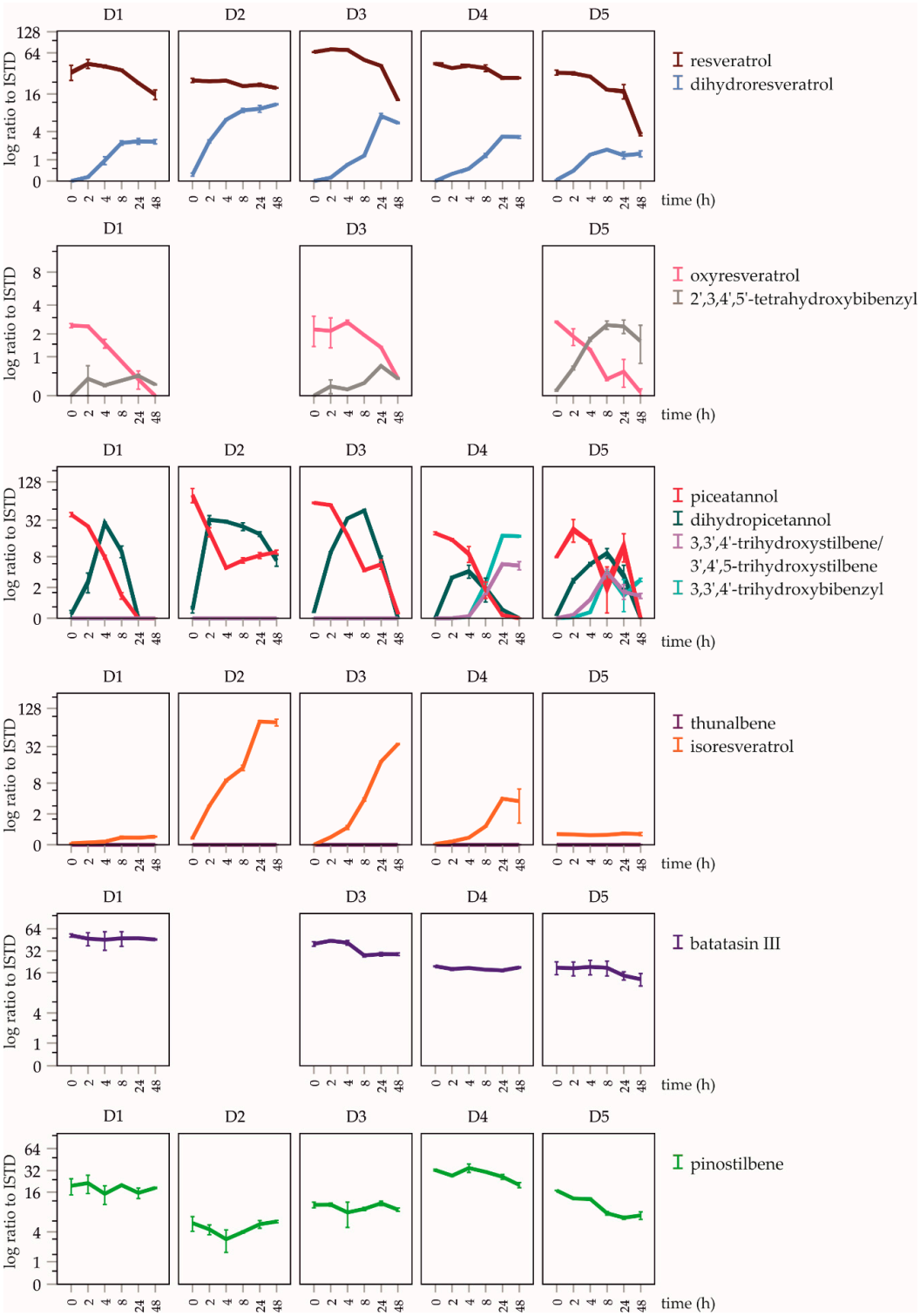


**Figure A2.** Comparison of metabolism between donors. Values are expressed as Means ± 1 SE, n = 3, technical replicates.

## Appendix C

**Table A1.** List of the stilbenoids monitored and detected in the samples by LC/MS.

| Name | Molecular Formula | Neutral Molecule Exact Mass | Measured $[M-H]^-$ Exact Mass | Comparison with Standard |
|---|---|---|---|---|
| **monitored:** | | | | |
| thunalbene | $C_{15}H_{14}O_3$ | 242.0943 | 241.0865 | YES |
| pinostilbene | $C_{15}H_{14}O_3$ | 242.0943 | 241.0865 | YES |
| piceatannol | $C_{14}H_{12}O_4$ | 244.0736 | 243.0657 | YES |
| oxyresveratrol | $C_{14}H_{12}O_4$ | 244.0736 | 243.0657 | YES |
| batatasin III | $C_{15}H_{16}O_3$ | 244.1099 | 243.1021 | YES |
| resveratrol | $C_{14}H_{12}O_3$ | 228.0786 | 227.0708 | YES |
| lunularin | $C_{14}H_{14}O_2$ | 214.0994 | 213.0916 | YES |
| **detected:** | | | | |
| dihydroresveratrol | $C_{14}H_{14}O_3$ | 230.0943 | 229.0865 | YES |
| 2,3′,4,5′-tetrahydroxybibenzyl | $C_{14}H_{14}O_4$ | 246.0892 | 245.0814 | NO |
| dihydropiceatannol | $C_{14}H_{14}O_4$ | 246.0892 | 245.0814 | NO |
| trihydroxystilbene | $C_{14}H_{12}O_3$ | 228.0786 | 227.0708 | NO |
| 3,3′,4-trihydroxybibenzyl | $C_{14}H_{14}O_3$ | 230.0943 | 229.0865 | NO |
| isoresveratrol | $C_{14}H_{12}O_3$ | 228.0786 | 227.0708 | NO |

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**Sample Availability:** Samples of the compounds are not available from the authors.

Clinical and Translational Medicine

REVIEW Open Access

# The gut microbiome and response to immune checkpoint inhibitors: preclinical and clinical strategies

Jun Gong[1], Alexander Chehrazi-Raffle[2], Veronica Placencio-Hickok[1], Michelle Guan[1], Andrew Hendifar[1] and Ravi Salgia[3*]

**Abstract**

There is growing interest in identifying predictive biomarkers for inhibitors of programmed cell death protein 1 receptor (PD-1), programmed death ligand 1 (PD-L1), and cytotoxic T-lymphocyte associated protein 4 (CTLA-4). Given the links between the stool microbiota, anticancer immunosurveillance, and general health, the composition of the gut microbiome has recently undergone investigation as a biomarker for immunotherapy. In this review, we highlight published results from preclinical and clinical studies to date supporting a relationship between the gut microbiome and antitumor efficacy of immune checkpoint inhibitors. Despite the promising and hypothesis-generating findings that have been produced in this arena to date, there remain some inconsistencies amongst present data that may need to be resolved to contribute to further development. Among these, a better understanding of the immunomodulatory function of the microbiome, standardization in sampling, sequencing techniques, and data analysis, and ensuring uniformity across various aspects of study design are warranted in conducting future prospective studies seeking to validate the gut microbiome as a potential biomarker of response to checkpoint blockade.

**Keywords:** Gut microbiome, Commensal bacteria, Biomarkers, PD-1, PD-L1, CTLA-4, Immune checkpoint inhibitors

## Introduction

There are currently several programmed cell death protein 1 receptor (PD-1) and programmed death ligand 1 (PD-L1) inhibitors approved by the Food and Drug Administration (FDA) in the treatment of solid and hematologic cancers [1]. As the clinical development of PD-1/PD-L1 inhibitors continues to pick up considerable momentum, so does the search for predictive biomarkers for this class of immunotherapy. Among the earliest and most widely recognized predictive biomarkers is PD-L1 expression though its absence in tumors certainly does not preclude response to PD-1/PD-L1 blockade [2].

Tumor mutational burden (TMB) has also been shown to predict benefit from immune checkpoint inhibitors across several tumor types due to generation of immunogenic neoantigens arising from an increased burden of nonsynonymous mutations [3]. Tumors harboring mutations in DNA mismatch repair genes resulting in microsatellite instability (MSI) or DNA polymerases (*POLE*) represent other phenotypes with high mutational load that can predict response to checkpoint blockade [4].

There is also growing interest in identifying the immune-active properties of the tumor microenvironment (TME) that constitute an immunologically "hot" tumor in responders to PD-1/PD-L1 blockade, in contrast to the immunologically "cold" tumor [5, 6]. For example, the type, density, and location of tumor infiltrating lymphocytes (TILs) are features that have been associated with response to checkpoint inhibition [7]. The Immunoscore represents a composite score incorporating such features of the infiltrating immune cell population and has been prospectively validated in colorectal cancer as a reliable prognostic indicator

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with further investigations in other tumors as a predictor of response to checkpoint blockade [8]. Profiling of the T-cell repertoire to assess for T-cell clonality may also serve as potential predictor of response to checkpoint inhibition [7]. Furthermore, assessment of a panel of markers associated with immune-sensitive or immune-resistant tumor phenotypes through gene expression profiling such as the Tumour Inflammation Signature or PanCancer IO 360 assay have shown promise in identifying candidates to PD-1/PD-L1 blockade [7].

More recently, the gut microbiome has emerged as another potential predictor of response to immune checkpoint inhibitors. The microbiome and its association with general health has long been described, and the potential to confer health benefits on the host through direct and indirect manipulation of the intestinal microflora has been a subject of investigation for the past several decades [9]. Examples of such manipulation have included probiotics, which are live microorganisms or biotherapeutic products that when administered in adequate amounts confer a health benefit on the host, and prebiotics, which are substrates such as carbohydrates or animal nutrition that are selectively used by host microorganisms to confer a potential health benefit. *Clostridium butyricum*, for instance, is a probiotic that has been shown to possess immunotherapeutic properties in cancer and gastrointestinal disorders [10, 11]. Prebiotics and synbiotics, which is a mixture of prebiotics and probiotics, have also demonstrated putative beneficial effects in the treatment of a multitude of other health conditions [12]. There are other microbiome studies investigating the relationship between the intestinal microbiota and efficacy of anticancer therapies, in general, in cancers of the lung and other organs, and the reader is referred to recent reviews [13–15]. In this review, we summarize the available evidence to date supporting the stool microbiota in shaping response to checkpoint blockade and their utility as a predictive biomarker for cancer immunotherapy. Beyond highlighting putative immunomodulatory mechanisms, we provide a phylogenetic classification of organisms associated with checkpoint inhibitor response and a succinct study-by-study tabulation of findings to allow one to readily compare results across preclinical and clinical studies. We also provide a novel discussion of inconsistencies across preclinical and clinical studies that does not serve to discredit the biomarker potential of the gut microbiome for checkpoint blockade, but rather to highlight areas in need of further investigation to strengthen the development of this exciting concept in immunotherapy.

## Preclinical studies

### CpG-oligonucleotides and anti-interleukin antibodies

An eloquent study involving mice subcutaneously injected with melanoma (B16) and colon carcinoma (MC38) cells pretreated with an antibiotic cocktail was among the first to show the relationship between the stool microbiome and response to immunotherapy [16]. Antibiotic-treated and germ-free mice showed significantly shorter survival and less tumor volume reduction with immunotherapy through injections of CpG-oligonucleotides and anti-interleukin (IL)-10 antibodies, when compared to controls, and highlighted that commensal gut microbiota primed tumor-infiltrating myeloid-derived cells through Toll-like receptor 4 (TLR4) activation and produce cytokines such as tumor necrosis factor (TNF) critical to antitumor efficacy (Table 1). Notably, administration of cultured *Allstipes* species (spp., *A. shahii*) or *Lactobacillus* spp. by gavage reconstituted or attenuated TNF-dependent tumor response to immunotherapy in antibiotic-treated mice, respectively (Table 1). Numbers of *Lactobacillus* spp. recovered as early as 1 week after stopping antibiotics, but recovery of *Allstipes* and *Ruminococcus* spp. was delayed, taking up to 4 weeks after stopping antibiotics.

### Anti-CTLA-4 antibodies

In a subsequent study, tumor-bearing mice housed in germ-free conditions or treated with antibiotics experienced comprised antitumor effects with anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) therapy that were associated with significantly decreased effector CD4+ T-cells and tumor-infiltrating lymphocytes (TILs), when compared to controls [17]. Oral feeding of these mice with various *Bacteroides* spp. or *Burkholderia* spp. restored response to anti-CTLA-4 therapy associated with T-helper 1 ($TH_1$) immune responses in tumor-draining lymph nodes and maturation of intratumoral dendritic cells (DCs, Table 1). Fecal transplantation studies from metastatic melanoma patients to tumor-bearing, germ-free mice treated with anti-CTLA-4 therapy demonstrated abundance of *Bacteroides* spp. that correlated with response. Intestinal reconstitution of antibiotic-treated mice with *Bacteroides fragilis* and *Burkholderia cepacia* was also shown to reduce anti-CTLA-4-induced colitis.

### Anti-PD-L1 antibodies

In mice subcutaneously injected with melanoma and bladder cancer, response to anti-PD-L1 therapy was significantly correlated with *Bifidobacterium*-treated mice (oral gavage) compared to non-*Bifidobacterium*-treated mice that was associated with increases in interferon γ (IFN-γ)-producing tumor-antigen-specific

**Table 1 Published preclinical studies investigating the relationship between gut microbiota and antitumor efficacy of immunotherapy**

| Model | Microbial intervention | Immunotherapy | Findings | References |
|---|---|---|---|---|
| MC38 and B16 tumor-bearing mice | Vancomycin, imipenem/cilastatin, neomycin in drinking water<br>Oral gavage | IT CpG-ODN and IP anti-IL-10 Abs | Impaired TNF-dependent antitumor activity and worse survival in antibiotic-treated and germ-free mice<br>Number of *Allstipes* and *Ruminococcus* spp. positively correlated while *Lactobacillus* spp. negatively correlated with TNF-dependent tumor response to immunotherapy | [16] |
| MCA205, Ret, and MC38 tumor-bearing mice | Ampicillin, colistin, and streptomycin or imipenem<br>Oral gavage<br>Fecal transplantation | IP anti-CTLA-4 Ab | Impaired antitumor activity in germ-free or antibiotic-treated mice but not in specific pathogen-free mice<br>In antibiotic-treated mice, oral feeding with *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, or *Burkholderia cepacia* recovered anti-CTLA-4 response<br>In germ-free mice, oral feeding with *Bacteroides fragilis* recovered anti-CTLA-4 response<br>Abundance of *Bacteroides* spp. (not *B. distasonis* or *B. uniformis*) correlated with response in human to mice fecal transplantation studies | [17] |
| B16.SIY and MB49 tumor-bearing mice | Oral gavage | IP anti-PD-L1 Ab | *Bifidobacterium* spp.-treated mice had significantly improved tumor control vs. non-*Bifidobacterium*-treated mice | [18] |
| BP tumor-bearing mice | Fecal transplantation | IP anti-PD-L1 Ab | Responders had significantly higher abundance of *Faecalibacterium* spp. and Ruminococcaceae family, while nonresponders had higher abundance of Bacteroidales order | [19] |
| MCA205, LLC, and Ret tumor-bearing mice | Ampicillin, colistin, and streptomycin<br>Fecal transplantation<br>Oral gavage | IP anti-PD-1 Ab ± anti-CTLA-4 Ab | Worse survival in antibiotic-treated and specific pathogen-free mice<br>Reconstitution with *Akkermansia muciniphila* ± *Enterococcus hirae* or *Alistipes indistinctus* reversed resistance to PD-1 blockade in antibiotic-treated mice | [20] |
| B16.SIY tumor-bearing mice | Fecal transplantation | IP anti-PD-L1 Ab | 2/3 mouse cohorts reconstituted with R fecal material showed slower baseline tumor growth<br>2/3 mouse cohorts reconstituted with NR fecal material showed faster baseline tumor growth<br>Anti-PD-L1 efficacy seen in mice colonized with R microbiota vs. completely ineffective in NR-derived mice | [21] |

*MC38* colon carcinoma, *B16* melanoma, *ODN* oligodeoxynucleotides, *IT* intratumoral, *IP* intraperitoneal, *IL-10* interleukin-10, *Abs* antibodies, *TNF* tumor necrosis factor, *spp.* species, *MCA205* sarcoma, *Ret* melanoma, *B16.SIY* melanoma, *CTLA-4* cytotoxic T-lymphocyte associated protein 4, *B16.SIY* melanoma, *MB49* bladder cancer, *PD-L1* programmed death ligand 1, *LLC* Lewis lung carcinoma, *PD-1* programmed cell death protein 1, *B16.SIY* melanoma, *R* responder, *NR* nonresponder

T-cells, major histocompatibility complex (MHC) Class II dendritic cells, and upregulation of gene transcripts involved in CD8+ T-cell activation and costimulation, DC maturation, antigen processing and cross presentation, chemokine-mediated immune cell recruitment to the TME, and type I interferon signaling [18]. Of note, *Bifidobacterium* was not detected in mesenteric lymph nodes, spleen, or tumor suggesting that systemic antitumor immune responses occurred independently of bacterial translocation.

In a separate melanoma-bearing mouse model, response to anti-PD-L1 therapy significantly correlated with fecal transplantations from patients abundant in Ruminococcaceae family and *Faecalibacterium* spp., while nonresponders to PD-L1 blockade had abundance in stool Bacteroidales order (Table 1). Mice responsive to checkpoint inhibition had significantly higher levels of CD8+ TILs and TME PD-L1 expression but lower levels of CD11b+CD11c+ suppressive myeloid cells compared to nonresponders, while increases in RORγT+ Th17 tumor-infiltrating cells and regulatory CD4+ FoxP3+ T-cells and CD4+ IL-17+ T-cells were observed in nonresponders [19].

#### Anti-PD-1 antibodies

In mice established with sarcoma and melanoma, 2 weeks of broad-spectrum antibiotics and rearing in specific pathogen-free conditions adversely affected survival with PD-1 ± CTLA-4 blockade [20]. Reconstitution with commensals such as *A. muciniphila* and *E. hirae* reversed resistance to PD-1 blockade in antibiotic-treated mice (Table 1). Interestingly, reconstitution with immune-sensitizing microbes was associated with accumulation of memory CCR9-expressing Th1-associated chemokine receptor-expressing CD4+ T-cells in tumor beds, metastatic lymph nodes, and draining lymph nodes 48 h after the first injection of anti-PD-1 antibody, formation of intratumoral granulomas, DC-induced IL-12 secretion, and increased CD4/Foxp3 ratios.

In a recent study involving fecal transplantation from melanoma patients who were responders and nonresponders to anti-PD-1 therapy into melanoma-bearing germ-free mice, anti-PD-L1 therapy was effective in mice colonized with responder microbiota and ineffective in mice colonized with nonresponder microbiota [21]. Responder microbiota-reconstituted mice had significantly higher numbers of SIY-specific $CD8^+$ T cells, but not $FoxP3^+CD4^+$ regulatory T cells in the TME compared to nonresponder-derived mice.

## Clinical studies

#### Baseline gut microbiome diversity

Numerous clinical studies investigating the stool microbiome in patients treated with checkpoint inhibitors have since been conducted in an attempt to corroborate findings demonstrated in preclinical models (Table 2). A prospective study collected buccal and fecal samples from 112 patients with metastatic melanoma prior to treatment with anti-PD-1 therapy [19]. Responders to anti-PD-1 therapy were significantly associated with higher diversity of gut microbiome and enriched with a unique stool bacterial composition compared to nonresponders; these findings were not observed in the oral microbiome (Table 2). Univariate analyses identified that the strongest predictors of response to anti-PD-1 therapy were alpha diversity [intermediate hazard ratio (HR) 3.60, 95% confidence interval (CI 1.02–12.74); abundance of *Faecalibacterium* genus (HR 2.92, 95% CI 1.08–7.89), and abundance of Bacteroidales order (HR 0.39, 95% CI 0.15–1.03)] in the gut microbiome. Interestingly, a significant positive correlation between tumor-infiltrating CD8+ TILs and higher levels of CD4+ and CD8+ T-cells in the systemic circulation with preserved cytokine response and abundance of the *Faecalibacterium* genus, Ruminococcaceae family, and Clostridiales order in the gut was observed. Conversely, a nonsignificant negative association between abundance of the Bacteroidales order and CD8+ TILs was observed. Higher abundance of Bacteroides order in the gut was associated with higher systemic levels of regulatory T-cells (Tregs) and myeloid derived suppressor cells (MDSCs) with a blunted cytokine response.

A recent investigation collected baseline stool samples from 42 patients with metastatic melanoma prior to anti-PD-1 therapy [21]. After removing operational taxonomic units (OTUs) found in < 10% of samples and integration of 16S ribosomal RNA gene sequencing, metagenomic shotgun sequencing, and quantitative polymerase chain reaction (PCR), a selection of 10 spp. was produced with differential abundance in responders and nonresponders to PD-L1 blockade (Table 2). Fecal transplantation from responding and nonresponding patients into melanoma-inoculated mice treated with anti-PD-L1 therapy largely recapitulated outcomes and enrichment patterns seen in original donors.

#### Effects of antibiotics

Clinical studies have also brought to attention the potential influence of antibiotics on outcomes in patients treated with checkpoint inhibitors. In one study of

**Table 2 Published clinical studies investigating the relationship between gut microbiota and antitumor efficacy of immunotherapy**

| Study | Tumor (n) | Checkpoint inhibitor | Findings | References |
|---|---|---|---|---|
| PS | Metastatic melanoma (n = 43) | Anti-PD-1 therapy (agent and dose not specified) | Higher diversity of gut microbiome in R (n = 30) vs. NR (13, $p < 0.01$), not observed in oral microbiome<br>Enrichment of Clostridiales order/Ruminococcaceae family in R vs. enrichment of *Bacteroides thetaiotaomicron*, *Escherichia coli*, and *Anaerotruncus colihominis* in NR, not observed in oral microbiome<br>Median PFS undefined with high vs. 242 days with low abundance of *F*aecalibacterium genus ($p = 0.03$); median PFS 188 days with high vs. median PFS 393 days with low abundance of Bacteroidales order ($p = 0.05$) | [19] |
| RS | Advanced NSCLC (n = 60), RCC (n = 40) | Anti-PD-1 therapy (agent and dose not specified) | *Akkermansia muciniphila* was significantly enriched in R vs. NR (validated in subsequent cohort with 27 NSCLC and 26 RCC pts)<br>In the NSCLC cohort, *A. muciniphila* was also enriched in R ($p = 0.045$ with/without antibiotics, $p = 0.026$ excluding antibiotic-treated) along with *Ruminococcus* spp., *Alistipes* spp., *Eubacterium* spp., *Bifidobacterium adolescentis*, *Bifidobacterium longum*, and *Parabacteroides distasonis* | [20] |
| PS | Metastatic melanoma (n = 26) | I3 or 10 mg/kg Q3 weeks → maintenance Q12 weeks | In 7 pts with immune-related colitis, significant reductions from baseline to time of colitis seen in Firmicutes phylum (*Ruminococcus*, Lachnospiracea incertae sedis, *Blautia*, *Clostridium* IV, *Eubacterium*, unclassified Lachnospiraceae and Pseudoflavonifractor) and colitis associated with decreased bacterial diversity<br>Baseline enrichment in *Faecalibacterium* genus and Firmicutes phylum (unclassified Ruminococcaceae, Clostridium XIVa and Blautia) significantly associated with longer PFS ($p = 0.0039$) and OS ($p = 0.051$) vs. *Bacteroides* spp. (independent of clinical characteristics)<br>Baseline enrichment with Firmicutes phylum significantly associated with developing colitis ($p = 0.009$) vs. Bacteroidetes phylum in those who did not develop colitis ($p = 0.011$) | [24] |
| PS | Metastatic melanoma (n = 39) | I3 mg/kg Q3 weeks X4 doses, N 1 mg/kg + I3 mg/kg Q3 weeks X4 doses → N 240 mg Q2 weeks, N 240 mg Q2 weeks, or P 2 mg/kg Q3 weeks | In all pts: Baseline enrichment with *Bacteroides caccae* ($p = 0.032$ and *Streptococcus parasanguinis* ($p = 0.048$) in R vs. NR<br>In N + I arm: Baseline enrichment with Firmicute phylum (*Faecalibacterium prausnitzii* ($p = 0.032$) and *Holdemania filiformis* ($p = 0.043$)) and Bacteroidetes phylum [*Bacteroides thetaiotamicron* ($p = 0.046$)] in R vs. NR<br>In P arm, baseline enrichment with *Dorea formicigenerans* ($p = 0.045$) in R vs. NR<br>In all pts, 83 gut metabolites at baseline were significantly different in R vs. NR (49 increased, 34 decreased, $p < 0.05$) | [25] |

**Table 2 (continued)**

| Study | Tumor (n) | Checkpoint inhibitor | Findings | References |
|---|---|---|---|---|
| RS | Locally advanced or metastatic NSCLC (n = 15) | N 3 mg/kg Q2 weeks | 73.3% received antibiotic monotherapy, 53.3% antibiotic duration > 7 days, 53.3% received antibiotics 1–3 months before first N, 33.4% < 1 month, and 13.3% during N<br>Rate of CR 26.7%, SD 33.3%, PD 40% in antibiotic-treated vs. rate of CR 22%, SD 27.1%, PD 50.9% in non-antibiotic-treated (p = 0.75)<br>No impact of antibiotics on PFS under N (p = 0.72) | [22] |
| RS | Advanced RCC (n = 16) or NSCLC (n = 48) | Anti-PD-1 or anti-PD-L1 antibody ± anti-CTLA-4 antibody (agents and dose not specified) | In RCC, PD rate 75% vs. 22% (p < 0.01), median PFS 1.9 vs. 7.4 months (HR 3.1, 95% CI 1.4–6.9, p < 0.01), median OS 17.3 vs. 30.6 months (HR 3.5, 95% CI 1.1–10.8, p = 0.03) in antibiotic-treated vs. no antibiotics (up to 30 days)<br>In NSCLC, PD rate 52% vs. 43% (p = 0.26), median PFS 1.9 vs. 3.8 months (HR 1.5, 95% CI 1.0–2.2, p = 0.03), median OS 7.9 vs. 24.6 months (HR 4.4, 95% CI 2.6–7.7, p < 0.01) in antibiotic-treated vs. no antibiotics (up to 30 days) | [23] |
| RS | Metastatic melanoma (n = 42) | Anti-PD-1 or anti-CTLA-4 therapy (agent and dose not specified) | 8 spp. more abundant at baseline in R: *Enterococcus faecium*, *Collinsella aerofaciens*, *Bifidobacterium adolescentis*, *Klebsiella pneumoniae*, *Veillonella parvula*, *Parabacteroides merdae*, *Lactobacillus* spp., and *Bifidobacterium longum*<br>2 spp. more abundant at baseline in NR: *Ruminococcus obeum* and *Roseburia intestinalis* | [21] |

*PS* prospective study, *PD-1* programmed cell death protein 1 receptor, *R* responders per the response evaluation criteria in solid tumors (RECIST 1.1) criteria, *NR* nonresponders, *PFS* progression-free survival, *RS* retrospective study, *NSCLC* non-small cell lung cancer, *RCC* renal cell carcinoma, *pts* patients, *I* ipilimumab, *Q* every, *OS* overall survival, *N* nivolumab, *P* pembrolizumab, *CR* complete response, *SD* stable disease, *PD* progression disease, *HR* hazard ratio, *CI* confidence interval

249 patients with advanced non-small cell lung cancer (NSCLC, n = 140), renal cell carcinoma (RCC, n = 67), and urothelial carcinoma (n = 42) treated with PD-1/PD-L1 blockade after ≥ 1 prior therapies, treatment with antibiotics (beta-lactam inhibitors, fluoroquinolones, or macrolides) 2 months before or 1 month after PD-1/PD-L1 blockade was significantly associated with shorter progression-free survival (PFS) and overall survival (OS) [20]. Shotgun sequencing identified an overrepresentation of bacterial genera most notably including *Akkermansia muciniphila* in responders to PD-1 inhibition compared to nonresponders (Table 2, with or without antibiotics). Only Th1 and Tc1-cell reactivity against *A. muciniphila* and IFN-γ production above median were significantly associated with PFS in patients treated with PD-1 antibody. Oral gavage of sarcoma-carrying mice with stool samples from NSCLC patients who were responders and nonresponders recapitulated sensitivity and resistance to PD-1 blockade, respectively.

One retrospective study of patients with locally advanced or metastatic NSCLC investigated the outcome of patients treated with nivolumab in the setting of antibiotic exposure [22]. Out of 15 patients treated with antibiotics, response and PFS was not significantly different among those receiving nivolumab exposed and not exposed to antibiotics (Table 2). This study contradicts that of a larger retrospective study assessing the benefit of checkpoint blockade in advanced RCC and NSCLC patients exposed to antibiotics up to 30 or 60 days before the first dose of checkpoint inhibitor [23]. Increased rates of progressive disease (PD), shorter PFS, and shorter OS were observed in RCC patients exposed to antibiotics up to 30 days, and shorter PFS and OS were observed in NSCLC patients exposed to antibiotics up to 30 days (Table 2). Results were largely similar on analysis of RCC patients exposed to antibiotics up to 60 days before first dose of checkpoint inhibitor. Although antibiotic use and tumor burden were independently associated with worse PFS but not OS on multivariate analysis in the RCC cohort, antibiotic use was independently associated with worsened OS in the NSCLC cohort.

#### Immune-mediated colitis

Clinical studies have also recently begun to describe the influence of the microbiota in modulating a unique toxicity of checkpoint blockade—immune-mediated colitis. In a prospective cohort of metastatic melanoma patients treated with ipilimumab, serial fecal samples were collected [24]. Relative reductions in gut microbiota were observed from baseline to time of onset of immune-related colitis in various members of Firmicutes phylum. Interestingly, baseline enrichment with Firmicutes phylum was significantly associated with developing colitis ($p = 0.009$) while significant enrichment in Bacteroidetes phylum was seen in those who did not develop colitis ($p = 0.011$). Patients who developed ipilimumab-induced colitis had significantly higher numbers of CD4+ T-cells but lower levels of IL-6, IL-8, and sCD25 at baseline compared to those without colitis. Notably, the investigators showed that antibiotics before ipilimumab treatment did not influence baseline dominant microbiota and none of the potentially predictive taxa were associated with antibiotic use.

#### Baseline gut microbiota and metabolic signatures

A separate prospective cohort of 39 metastatic melanoma patients, of which 8% had used antibiotics prior to and/or during checkpoint blockade and 3% used probiotics, underwent metagenomic and metabolomic shotgun sequencing and provided a snapshot of baseline or pretreatment gut microbiota signatures associated with response to checkpoint inhibitors as well as significantly enriched and depleted metabolites involved in numerous metabolic pathways in responder metabolomes (Table 2) [25].

## Discussion

The list of potential biomarkers that predict response, or lack of through primary, adaptive, and acquired resistance, to checkpoint inhibitors is growing [26]. In the past 5 years, research into the association between the gut microbiome and response to PD-1/PD-L1/CTLA-4 inhibitors has produced interesting findings on the topic (Tables 1, 2). The list of microbes that have been positively correlated with response to checkpoint blockade in the preclinical realm include: *Bacteroides* spp. and *Burkholderia* spp. (anti-CTLA-4), *Bifidobacterium* spp., *Faecalibacterium* spp., and more broadly, Ruminococcaceae family (anti-PD-L1), and *Akkermansia muciniphila*, *Alistipes indistinctus* (of the Bacteroidales order), and *Enterococcus hirae* (anti-PD-1, Table 1). However, abundance of stool Bacteroidales order (includes *Bacteroides* spp.) has been associated with nonresponders to anti-PD-L1 therapy in a separate preclinical study [19].

In clinical studies, findings that are both concordant and discordant to other clinical and preclinical studies on the gut microbiome have been produced (Table 2). Enrichment in the Firmicutes phylum (includes the Clostridiales order, e.g., *Dorea formicigenerans*, *Eubacterium* spp., and *Veillonella parvula*, Ruminococcaceae family, e.g., *Ruminococcus* spp., *Blautia* genus, *Faecalibacterium* genus, e.g., *Faecalibacterium prausnitzii*, and individual organisms *Enterococcus faecium*, *Holdemania filiformis*, *Lactobacillus* spp., and *Streptococcus parasanguinis*), *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Akkermansia muciniphila*,

*Collinsella aerofacien*, *Klebsiella pneumoniae*, *Alistipes* spp. (of the Bacteroidales order), and *Parabacteroides merdae*/*distasonis* (of the Bacteroidales order) have been associated with response to PD-1 and CTLA-4 blockade in humans (Fig. 1) [19–21, 24, 25], while Bacteroidales order (includes *Bacteroides* spp., e.g., *Bacteroides thetaiotaomicron*), *Escherichia coli*, and *Anaerotruncus colihominis* (of the Clostridiales order/Ruminococcaceae family), and *Roseburia intestinalis* (of the Clostridiales order) have been negatively associated with response to anti-PD-1 and anti-CTLA-4 therapy [19, 21, 24]. Notably, baseline enrichment in Bacteroidetes phylum (includes *Bacteroides thetaiotamicron* and *Bacteroides caccae*) has been associated with response to anti-PD-1 and anti-CTLA-4 therapy in melanoma patients [25], which is in contrast to some preclinical and clinical evidence described previously that support their abundance as associated with lack of response. Furthermore, lack of response to anti-PD-1 or anti-CTLA-4 therapy in another melanoma cohort has been associated with baseline abundance in *Ruminococcus obeum*, which contradicts other preclinical/clinical data supporting that gut enrichment with Ruminococcaceae family and *Ruminococcus* spp. positively correlate with response to checkpoint inhibitors [21].

Where the utility of the stool microbiota falls along the spectrum of clinically-relevant biomarkers for checkpoint blockade is unclear given the incongruent findings present in both published preclinical and clinical studies to date. Although interesting and thought-provoking, there remain a number of critical issues at hand that need to be addressed in order to establish the candidacy of the gut microbiome as a predictive biomarker for this promising class of immunotherapy.

## Immunomodulatory mechanisms

It has long been implicated that the microbiome is involved in tumorigenesis as well as activation or suppression of the immune system that can contribute to tumor control or escape [27, 28]. Early attempts in linking the gut microbiome and anticancer immunosurveillance hypothesized that (1) microbial antigens may sufficiently stimulate antitumor immune activity through tumor antigenic mimicry or cross-reactivity, (2) microbes may provide a non-antigenic co-stimulus or secondary signal (or collection of signals) resulting in bystander activation of tumor associated antigen-specific T lymphocytes, and/or (3) microbial toxins and byproducts may directly or indirectly (through immunosurveillance) affect cancer cells [28]. Specific to the antitumor activity of immune checkpoint inhibitors, a growing body of evidence now posits that the gut microbiota may enhance the function of DCs with more potent tumor antigen presentation and cytokine production, increase trafficking of CD4+ memory T-cells from mesenteric and draining lymph nodes to the TME, decrease Tregs and MDSCs, and increase recruitment and activation of IFN-γ-producing tumor-antigen-specific effector T-cells that

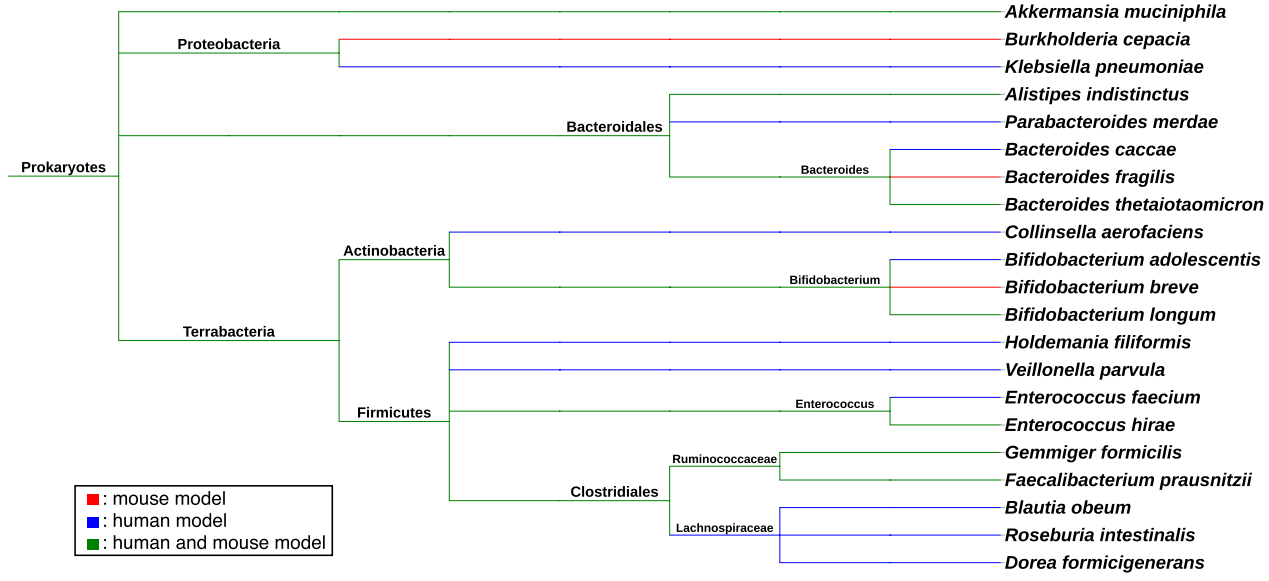


**Fig. 1** Phylogenetic tree of gut commensal bacteria associated with response to immune checkpoint inhibitors in both preclinical and clinical studies. Taxonomic classification is based on (from left to right) domain, phylum, order, family, genus, and species except for Terrabacteria (unranked). Figure created using the phylogenetic tree software by: [44]

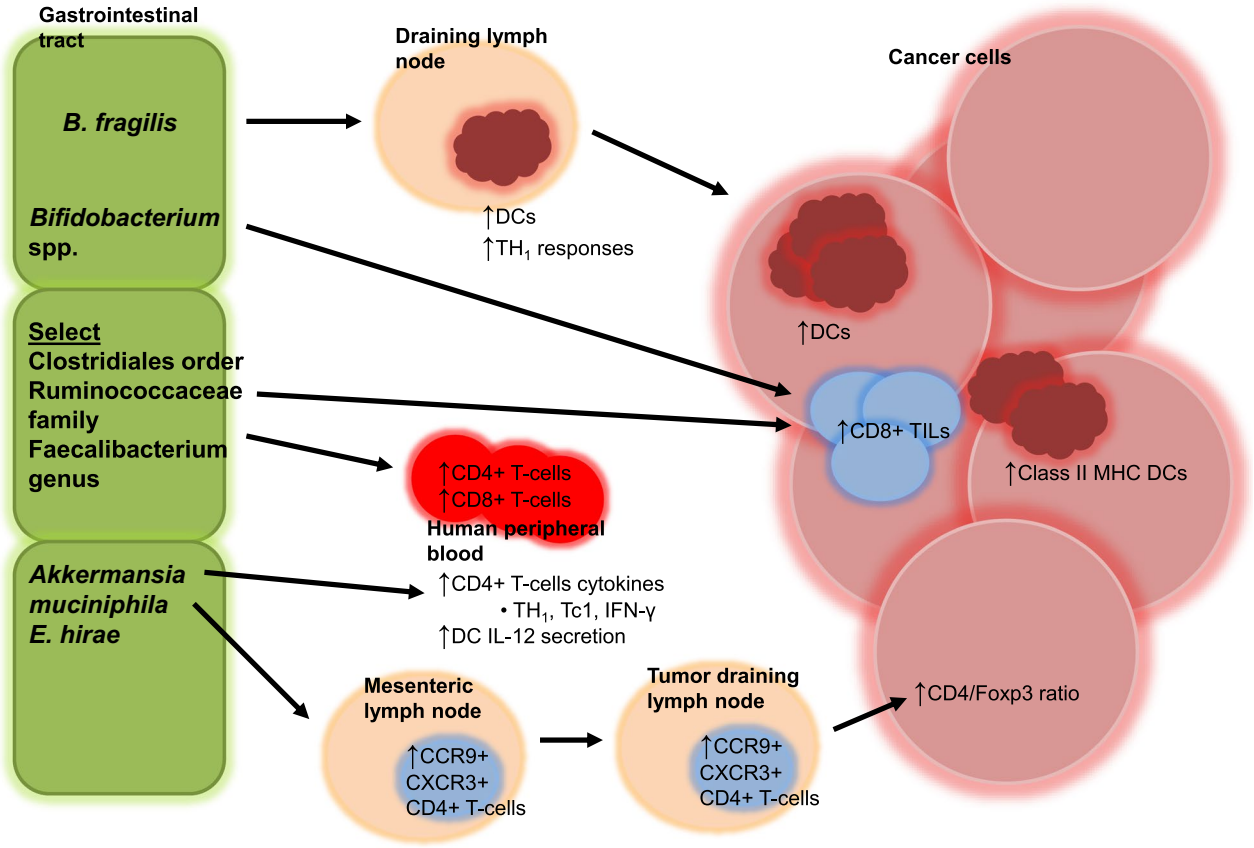


**Fig. 2** Proposed immunomodulatory mechanisms of commensal bacteria on anticancer efficacy of immune checkpoint inhibitors in animal models and patients. Oral gavage of B. fragilis in germ-free mice has been shown to induce T helper 1 ($TH_1$) immune responses in tumor-draining lymph nodes and maturation of dendritic cells (DCs) in responders to cytotoxic T-lymphocyte associated protein 4 (CTLA-4) blockade. Oral gavage of Bifidobacterium spp. in mice was shown to increase accumulation of antigen-specific $CD8^+$ tumor-infiltrating lymphocytes (TILs) and major histocompatibility complex (MHC) Class II DCs in responders to programmed death ligand 1 (PD-L1) blockade. Human responders to programmed cell death protein 1 receptor (PD-1) blockade had significant positive correlations between CD8+ TILs or levels of CD4+ and CD8+ T-cells in the peripheral blood and abundance of select members of the Clostridiales order, Ruminococcaceae family, and *Faecalibacterium* genus. Oral gavage of *Akkermansia muciniphila* and *E. hirae* was associated with increased central memory CD4+ T-cells expressing the small intestine-associated chemokine receptor CCR9 and/or the $TH_1$-associated chemokine receptor CXCR3 in mesenteric and tumor draining lymph nodes as well as increased CD4/Foxp3 ratios in tumors of mice cotreated with anti-PD-1 therapy. In human peripheral blood, secretion of cytokines by CD4+ T-cells including $TH_1$, Tc1, and interferon-γ (IFN-γ) and bone marrow-derived DCs including IL-12 were associated with response to PD-1 blockade and reactivity against *A. muciniphila* and *E. hirae* (for Tc1)

altogether contribute to the modulation of the antitumor immune response (Fig. 2) [29].

Evidence is also accumulating to support that immunoregulatory pathways that facilitate checkpoint inhibitor response may be commensal-specific [30]. In preclinical models, inoculation of mice with *B. fragilis*, *A. muciniphila*, and *E. hirae* have been shown to induce $TH_1$ immune responses, promote maturation of DCs, and increase central memory CD4+ T-cells in mesenteric lymph nodes, tumor draining lymph nodes, and/or the TME in response to checkpoint inhibitors [17, 20]. Oral gavage of *Bifidobacterium* spp. in mice cotreated with anti-PD-L1 therapy was shown to increase antigen-specific CD8+ TILs and MHC Class II DCs, while abundance of Clostridiales order, Ruminococcaceae family, and *Faecalibacterium* genus was associated with increased CD8+ TILs and peripheral blood CD4+/CD8+ T-cells in human responders to PD-1 blockade [18, 19]. Abundance of *A. muciniphila* and *E. hirae* has been shown to be associated with secretion of cytokines by MCH Class II-restricted CD4+ T-cells and DCs in the peripheral blood of human responders to PD-1 blockade (Fig. 2) [20].

Despite the initial insights into the immunomodulatory mechanisms of the stool microbiome, the exact mechanisms linking commensal bacterial species to the anticancer efficacy of checkpoint blockade in animal

models and humans remain elusive. Our understanding of the impact of gut commensals on checkpoint inhibitor response has benefited greatly from experiments performing immune profiling in subjects treated with immunotherapy and inoculated with specific bacteria [17–20]. Further insights into direct cause-effect relationships between checkpoint inhibitor response and stool microbiota have been afforded by fecal transplantation from human responders of immunotherapy to mice with in-depth characterization of immune responses [19–21]. However, these studies did not further identify the specific bacteria whose abundance was associated with immune responses; in recognition that fecal transplantation from human responders can contain a diversity of microbials and that mechanisms of checkpoint inhibitor response can be commensal-specific, broader investigation involving inoculation with single-lineage bacteria and immune profiling in responders would be prudent in our understanding of gut microbiome-facilitated response to immunotherapy.

An overarching question in this area is whether the abundance of stool bacteria associated with response to checkpoint blockade is simply a reflection of the presence of health-associated bacteria that are of usual higher quantities in healthier individuals with more robust and functional immune systems or is it through mechanisms of the bacteria themselves that determine the host immune system's capability to engage in antitumor responses [30]. On this latter note, it should also be asked whether the antitumor immune response is dependent solely on bacterial properties and their direct interactions with the immune checkpoint inhibitors or through interactions involving the host-bacterial ecosystem and immunomodulatory cells [31]. Another research strategy to improve our understanding in this arena could entail investigating the magnitude by which gut commensals themselves stimulate innate and adaptive antitumor immune responses; these analyses have been initially presented in several studies [17, 18, 20]. Future study in controlled experiments evaluating immune profiles from inoculation of stool microbiota with and without checkpoint inhibitors could provide further understanding of (1) whether immune response pathways elicited by commensals are distinct from those generated by checkpoint blockade in altogether providing synergistic antitumor activity or (2) whether checkpoint blockade elicits antitumor responses that overlap the same immune response pathways activated in recognition of bacterial antigens and byproducts. Additionally, greater understanding of underlying mechanisms may be afforded in research on the contribution of the microbiome to therapy-induced anticancer immune responses across other treatment modalities beyond checkpoint inhibition such as chemotherapy, radiation therapy, hematopoietic stem cell transplantation, and other forms of immunotherapy [32, 33].

Furthermore, metabolomics analysis has recently identified significant differences in 83 gut metabolites at baseline in responders to anti-PD-1 and anti-CTLA-4 therapy compared to nonresponders with metastatic melanoma [25]. In essence, bacterial metabolites and byproducts of metabolic pathways involved in amino acid metabolism, lipid metabolism, nucleotide metabolism, and carbohydrate metabolism may also affect response to checkpoint blockade. As the putative mechanisms by which commensal bacteria facilitate response to immunotherapy increases in complexity, further understanding of the relationships between the gut microbiome and the antitumor immune response is critical in predicting success to checkpoint blockade.

#### Translation from preclinical to clinical settings

As stated previously, several inconsistencies in the gut microbiome composition have been produced in recent preclinical and clinical studies focused on investigating the relationship between stool microbiota and response to checkpoint inhibition (Tables 1, 2). Beyond associations between specific commensals and response (or lack of) to checkpoint blockade, increased representation of baseline Bacteroidetes phylum (includes *Bacteroides fragilis*) in melanoma patients and intestinal reconstitution with *Burkholderia cepacia* in antibiotic-treated, tumor-bearing mice have been shown to reduce anti-CTLA-4-induced colitis potentially by limiting inflammation through stimulation of Treg differentiation [17, 24, 34]. This is in contrast to studies showing an association with colonization by *Bacteroides* spp. and ulcerative colitis and Crohn's disease in mice models and humans [35–38]. Moreover, antibiotic use has been correlated with poorer outcome in tumor-carrying mice and metastatic RCC and NSCLC patients treated with anti-PD-1 and anti-CTLA-4 antibodies [17, 20, 23]. However, in 1 prospective cohort of metastatic melanoma patients treated with ipilimumab and 1 retrospective cohort of advanced NSCLC patients treated with nivolumab, antibiotic use had no impact on response to checkpoint blockade or association on potentially predictive taxa [22, 24]. Lastly, a higher diversity of the gut microbiome in responding patients with melanoma to anti-PD-1 therapy was observed compared to nonresponders [19]. However, a separate melanoma cohort identified that there were no significant differences in the level of gut microbial diversity between responders and nonresponders to anti-PD-1 and anti-CTLA-4 therapy [25].

These inconsistencies across preclinical and clinical studies highlight several important points that need to be

considered in development of future research in this area. Firstly, caution should be taken in extrapolating data from mice studies into humans. The anatomical structures and intestinal wall linings have been shown to significantly differ across human and mouse gastrointestinal tracts [39]. It has also been shown that 85% of the bacterial genera found in the mouse gut microbiome is not present in humans [40]. Furthermore, dynamic shifts in microbial species distribution can often occur due to host diet or lifestyle as well as interspecies competitive exclusion [31, 41]. Sampling and sequencing technique of stool specimens is another factor that can introduce variability in correlating the composition of the gut microbiome with checkpoint inhibitor response. Most human gut microbiome studies utilize stool samples, while mouse gut microbiome studies usually rely on cecal contents unless pellets are sampled in some longitudinal studies [39]. Historically, the standard choice for mouse studies has been mostly 16S rRNA sequencing whereas human microbiome studies have used both metagenomic and 16S rRNA sequencing approaches [39]. Metagenomic shotgun sequencing has several potential advantages over 16S rRNA sequencing as it can eliminate PCR bias seen with taxa that are over- or underrepresented depending on the choice of primers and 16S rRNA variable region to be amplified, improve gut microbiome taxonomic resolution at the species level given that bacteria belonging to the same genus can have different phenotypes or host effects, and provide information on metabolic pathways of the microbiome [25]. Nevertheless, variability can exist in either strategy due to differences in collection, storage, and processing of stool samples, extraction protocols for nucleic acids, and approaches used in data analysis [30].

A third consideration encompasses study design, which has general applicability across models despite its particular relevance to non-preclinical studies. Differences in study design including retrospective vs. prospective design, sample size, experimental subject and tumor heterogeneity, and checkpoint inhibitor such as anti-CTLA-4 vs. anti-PD-1/PD-L1 can certainly account for the variability in findings across microbiome studies in animals and humans [31]. Differences in frequency of sampling can also affect the accuracy to describe variations in taxome distribution over time given that although the individual gut microbiome can remain stable for long durations of time, changes in composition of the microbiome can rapidly occur due to antibiotics, dietary, and environmental changes [25]. In the largest cohort to date investigating the impact of antibiotics on the gut microbiota and response to checkpoint blockade, factors with potential impact on the microbiota composition such as diet, country of origin, and use of other medications were not taken into account [23]. It should be pointed out that although a detrimental effect of antibiotics on response to checkpoint blockade was identified in this study, the authors are unclear whether this reflects a general prognostic association or a causative link with resistance to checkpoint inhibition [23].

#### Future directions for clinical studies

The gold standard in designing the ideal investigation of the gut microbiome composition as a predictor of response to checkpoint blockade would involve taking into consideration all of the above points and incorporating them into a study of large sample size and prospective design. This is easier said than done, but to ensure our success in conducting high-quality research with minimal bias and confounding factors in this arena, future efforts can implement several key study parameters. Techniques in sampling and sequencing should be standardized; in the case of 16S rRNA sequencing, it will be important to minimize variations in the many proposed algorithms for clustering of genetic sequences into OTUs to measure microbiome diversity that have been found to have a negative influence on downstream analyses [31]. Furthermore, serial and longitudinal sampling will be of value to assess changes in an individual's gut microbiome over time in relation to checkpoint inhibitor response [25, 42]. To the best of our ability, controlling for or taking into account baseline differences in an individual's microbiome profile across patient demographics such as sex, age, race, comorbidities, medications including antibiotics and probiotics, diet and lifestyle, and environment/geographic location will add greatly to the development of a more standard measurement for future microbiome investigations [31].

It is increasingly understood that the diversity of the gut microbiome may include some bacterial species that are immunosuppressive while others that are immune-stimulatory [43]. Rather than risk the likelihood of underestimating the total number of bacteria showing differential abundance in responders compared to nonresponders of checkpoint inhibition (a problem often encountered in 16S rRNA sequencing given that the analysis is limited by the number of samples above the detection threshold), representing the data in aggregate through construction of a ratio comprised of the total number of "beneficial" and "nonbeneficial" OTUs has demonstrated feasibility in producing a composite commensal microbiota score that is predictive of benefit to checkpoint blockade [21]. Furthermore, improvements in the isolation of cultivable bacteria and derivation of individual clones with implementation of whole-genome sequencing may represent future steps in our ability to study the composition of the gut microbiome [30]. In developing the ideal biomarker for checkpoint inhibitors beyond the gut microbiome,

**Table 3 Ongoing select clinical studies investigating the effect of gut microbiota on anticancer therapies**

| Study | Tumor, setting | Interventions | Primary endpoint(s) | NCT |
|---|---|---|---|---|
| Observational, n = 49 | TNBC, newly diagnosed | Neoadjuvant chemotherapy with collection with pre- and post-therapy stool and PB samples | pCR rate as associated with composition of intestinal microbiota and subsequent short-term alterations in composition | NCT03586297 |
| Observational, n = 80 | Metastatic CRC, first-line; metastatic carcinoma, first-line anti-PD-1/PD-L1 therapy | FOLFOX or FOLFIRI or anti-PD-1/PD-L1 therapy with collection of pre-therapy and interval stool samples | Tumor response correlated with presence and amounts of species | NCT02960282 |
| Observational, n = 120 | AML, newly diagnosed or undergoing HSCT | Serial stool samples analyzed by next-generation sequencing | Association between changes in the intestinal microbiota and the incidence of gastrointestinal GVHD | NCT03148197 |
| Case–control, n = 200 | Glioblastoma multiforme, first-line | Concurrent chemoradiation (temozolomide) or radiation therapy or healthy control and collection of pre- and post-surgery stool samples | Pre-operative gut microbiome composition, perturbation of gut microbiota by temozolomide, and correlation of gut microbiota and prognosis | NCT03631823 |
| Phase I, n = 40 | Advanced melanoma, treatment refractory | FMT from responders of immunotherapy | Safety and comparison of gut microbiome composition pre- and post-FMT | NCT03353402 |
| Phase I/II, n = 20 | AML or high-risk MDS, first-line | Induction therapy + autologous FMT | Efficacy in dysbiosis correction by measure of microbiota diversity and eradication of MDRB | NCT02928523 |
| Phase II, n = 20 | Advanced melanoma, treatment refractory | FMT + pembrolizumab | ORR | NCT03341143 |
| Phase II, n = 144 | Any hematologic malignancy undergoing HSCT | Piperacillin–tazobactam or cefepime | Fold-change in Clostridiales abundance | NCT03078010 |

*NCT* ClinicalTrials.gov identifier, *TNBC* triple-negative breast cancer, *PB* peripheral blood, *CRC* colorectal cancer, *PD-L1* programmed death ligand 1, *PD-1* programmed cell death protein 1, *FOLFOX* 5-fluorouracil, leucovorin, and oxaliplatin, *FOLFIRI* 5-fluorouracil, leucovorin, and irinotecan, *AML* acute myeloid leukemia, *HSCT* hematopoietic stem cell transplantation, *GVHD* graft-versus-host disease, *FMT* fecal microbiota transplantation, *MDS* myelodysplastic syndrome, *MDRB* multidrug resistant bacteria, *ORR* overall response rate

future investigations may expand their attention beyond bacteria to the broader ecological community such as viruses and fungi; integration of the microbiome with metabolomics, proteomics, and genomics may provide an even more comprehensive prognostic and predictive biomarker [30, 42].

Lastly, with better uniformity across sampling techniques, data analysis, and study design and a greater understanding of the immunomodulatory mechanisms of the microbiome, we will be primed to investigate strategies to modify the gut microbiome and potentially improve cancer outcomes. There are numerous ongoing clinical studies and prospective trials investigating the role of intestinal commensals and their effect on anticancer therapies (Table 3). Ideally, these studies will provide some clarity to many of the questions that have emerged on manipulation of the stool microbiome and cancer immunotherapy. In line with the concept of precision oncology, a future goal would involve manipulation of an individual's microbiome through potential strategies including fecal microbial transplantation, provision of single bacterial species or a cocktail of beneficial organisms, dietary interventions, antibiotics, and/or probiotics to enhance the effect of anticancer therapies [30].

## Conclusion

Preclinical and clinical evidence is accumulating to support an association between the gut microbiome composition and antitumor efficacy of immune checkpoint inhibitors. However, to further its advancement as a potential biomarker for immunotherapy, there are several inconsistencies amongst present data that should be addressed. A greater understanding of the immunomodulatory mechanisms of the microbiome, standardization of sampling, sequencing techniques, and data analysis, and ensuring uniformity in study design are key considerations that may need to be incorporated into future investigations. Ultimately, validation of findings from existing preclinical and clinical data in subsequent studies of large sample size and prospective design is warranted to further develop the stool microbiota as a biomarker for checkpoint blockade.

#### Abbreviations

PD-1: programmed cell death 1; PD-L1: programmed death-ligand 1; CTLA-4: cytotoxic T-lymphocyte antigen 4; FDA: Food and Drug Administration; TMB: tumor mutational burden; MSI: microsatellite instability; POLE: DNA polymerase epsilon; TME: tumor microenvironment; TILs: tumor-infiltrating lymphocytes; B16: melanoma cell line; MC38: colon carcinoma cell line; IL: interleukin; TLR4: Toll-like receptor 4; TNF: tumor necrosis factor; spp.: species; $TH_1$: T-helper 1; DCs: dendritic cells; IFN-γ: in interferon γ; MHC: major histocompatibility complex; HR: hazard ratio; CI: confidence interval; Tregs: regulatory T-cells; MDSCs: myeloid derived suppressor cells; OTUs: operational taxonomic units (OTUs); PCR: polymerase chain reaction; NSCLC: non-small cell lung cancer; RCC: renal cell carcinoma; PFS: progression-free survival; OS: overall survival; PD: progressive disease.

#### Authors' contributions

JG, AC, VP, MG and AH: literature search and review, writing, graphical design, and editing; JG, AC, and RS: conception and design and editing. All authors read and approved the final manuscript.

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