

**FLAVOURS, FRAGRANCES AND INGREDIENTS**

*Essential Oils, Botanical Extracts, Cold Pressed Oils,  
Infused Oils, Powders, Flours, Fermentations*



**LITERATURE REVIEW HEALTH BENEFITS  
BOTANICAL INNOVATIONS  
APPLE CIDER VINEGAR POWDER  
& FERMENTED EXTRACTS**



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## LITERATURE REVIEW HEALTH BENEFITS APPLE CIDER VINEGAR AND APPLE CIDER VINEGAR POWDER

Botanical Innovations has developed a range of Naturally Fermented Vinegars and Extracts which combine great flavour with a range of health benefits. Dating back 5,000 BC vinegar has been a staple part of the human diets for millennium. The first recorded use was by the Babylonians who used vinegar as a condiment and preservative. The Ancient Romans consumed vinegar as a beverage and the Ancient Greeks used vinegar to pickle meat and vegetables. The healing properties of vinegar have also been referenced in the bible and the work of Hippocrates. By 2,000BC vinegar production became commercialized and was used to treat disease and wounds.

Today vinegar is used as in foods and beverages and as a health supplement. The potential benefits of Botanical Innovations.

Today vinegar is used taken orally and topically as a health supplement, in foods and beverages and as an ingredients in skin and hair care products.

This literature review summerizes recently published literature about the potential health benefits of Botanical Innovations Apple Cider Vinegar, Apple Cider Vinegar Powder with 'Mother', Fermented Vinegars and Grape Seed and Skin Extracts.

The literature identifies the following potential health benefits:

- Asthma symptom alleviation
- Diabetes prevention and treatment
- Antioxidant
- Lowers glucose intake
- Prevention of Cardiovascular disease
- Lowers cholesterol
- Contains anti glycemc properties
- Weight loss
- Lowers blood press
- Gut Health
- Prebiotic for intestinal health



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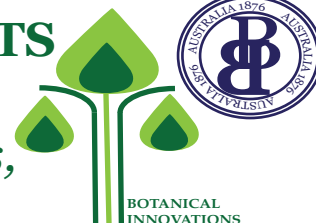
## **APPLE CIDER VINEGAR WITH 'MOTHER' POWDER A PREBIOTIC FLAVOUR NATURAL PRESERVATIVE**



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## APPLE CIDER VINEGAR POWDER WITH 'MOTHER' PREBIOTIC FLAVOUR NATURAL PRESERVATIVE

Botanical Innovations spray dried apple cider vinegar is a natural prebiotic flavouring and a natural preservative.

This versatile ingredient can be used to flavour chips, snacks and beverages providing a natural vinegar flavour. The product can also be added to variety of foods and beverages as a natural preservative for shelf life extension.

Botanical Innovations Apple Cider Vinegar Powder is a natural preservative made from 100% Australian apples. The vinegar is slowly fermented and spray dried concentrating the vinegar to provide food preservation properties including protection against mould, fungal growth and microbial contamination. Apple Cider Vinegar Powder also improves taste, allows for a clean labelling and has no impact upon product appearance.

Natural preservative applications include baked goods including bread, biscuits, snacks and beverages.

Prebiotics are a type of non-digestible carbohydrate found Botanical Innovations Apple Cider Vinegar, which promotes the growth and activity of good bacteria in the gut. Apple Cider Vinegar Powder is a prebiotic flavouring for snacks foods, potato and vegetable chips, functional foods, condiments, sauces, powder mixes, beverages and may be used as a standalone spice.

This is a versatile ingredient with a broad range of applications. Botanical Innovations Apple Cider Vinegar Powder is made from 100% Australian grown apples which is slowly naturally fermented and then spray dried creating a unique functional ingredient for food preservation, shelf life extension and prebiotic flavouring.

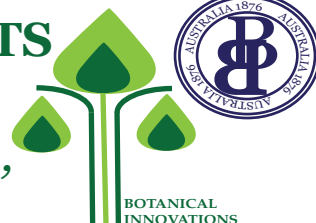
The combination of a functional flavouring ingredient with food preservation characteristics makes Botanical Innovations Apple Cider Vinegar Powder different to other solutions on the market. A number of natural preservatives currently available do not perform as well as artificial preservatives and or they have an impact upon the taste, mouthfeel, functionality of the product they are preserving. Botanical Innovations Apple Cider Vinegar Powder solves this problem in most applications. Apple Cider Vinegar Powder in baked goods if used as a preservative is flavour neutral and does not impact upon the taste, mouthfeel or functionality of the final product.

It has taken four years of research and development to perfect the flavour and functionality of Apple Cider Vinegar Powder and to ensure the product is able to act as both a natural preservative and prebiotic flavouring.



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## DATA SHEET

### PRODUCT

## RAW APPLE CIDER VINEGAR WITH 'MOTHER' POWDER

**APPLE CIDER VINEGAR POWDER** is a primary source of phenolics, prebiotic. This spray dried product is suitable for a variety of products.

### INGREDIENTS

Botanical Name: *Malus domestic*, *Malus pulila*  
CAS: 8028-52-2  
Naturally Fermented with 'Mother'  
Spray Dried Apple Cider Vinegar with Maltodextrin.

### CONCENTRATED PHYTONUTRIENTS

**APPLE CIDER VINEGAR POWDER** contains the following highly concentrated natural compounds

**Antioxidants:** Capture oxygen delaying oxidation which may prevent or delay cell damage.

**Phenols:** Large group of molecules found in plants. These compounds are responsible for the taste, colour and mouthfeel and are divided into phenolic acids and flavonoids.

**Prebiotics** are a type of non-digestible carbohydrate found in certain foods, which promote the growth and activity of good bacteria in the gut, Prebiotics assist nurturing the beneficial bacteria in your gut can improve the absorption of vitamins, support the guts natural immune defences and help support digestive balance.

January 2017. The information contained in this datasheet has been gathered from publically available source material and is intended to provide general information to readers. No therapeutic claim in relation to the product is intended. Whilst reasonable care has been taken in the preparation of this datasheet, General Industry Pty Ltd trading as Botanical Innovations is not responsible for any reliance readers place on the information in the datasheet and does not represent or warrant that the information in the datasheet is complete or accurate. Readers rely on the information in the datasheet at their own risk. Disclaimer: Reasonable care has been taken in preparing this document and the information provided herein is believed to be accurate. However, this information is not intended to constitute an "authoritative statement" under the National Industrial Chemical Notification and Assessment Scheme Australia and New Zealand rules and regulations.



### APPLE CIDER VINEGAR POWDER

### APPLICATIONS FLAVOUR & INGREDIENT PRIVATE LABEL

Differentiate your products with Apple Cider Vinegar Powder. Water Soluble suitable as a flavouring, ingredients for foods, beverages, natural healthcare supplements.

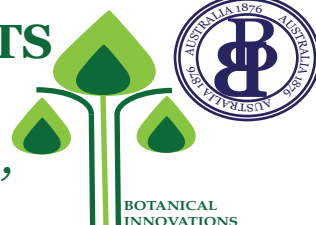
<b>Origin</b>	Australia
<b>Shelf Life</b>	Unopened 24 Months
<b>MOQ</b>	50kg



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## DATA SHEET PRODUCT AUSTRALIAN APPLE CIDER VINEGAR POWDER

### SPECIFICATIONS

Description:	White to Off White Fine Powder
Parts Used:	Apple
Flavour/Odour:	Characteristic Apple Vinegar
Organic Acid:	>37%
Soluble:	Water
Density:	40-60g/100ml
Loss on Drying:	<5%
Sulphate:	5ppm

Organic Acid (Citric and Malic Acid)	38%
Plant Starch	23%
Polyscharides	19%
Carrier Maltodextrin	20%

### NUTRITIONAL INFORMATION

	100 g
Energy	1250kj/299kcal
Protein	0
Fat	0
Saturated Fat	0
Carbohydrate	8g
Total	8g
Sugars - Total	2g
Sodium	1.3 mg

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### PACKAGING

20kg drums. For orders over 1 tonne contact us to discuss your requirements.

### SHELF LIFE

Shelf Life 2 years unopened.

### ALLERGENS

	Contains/ Potential Contamination
Cereals & cereal products + Gluten	No
Crustaceans & their products	No
Eggs & egg products	No
Fish & fish products	No
Peanuts or peanut products	No
Soybeans or their products	No
Milk or milk products	No
Nuts & nut products	No
Celery & celery products	No
Mustard & mustard products	No
Sesame seeds & sesame products	No
Sulphur dioxide & sulphites	No

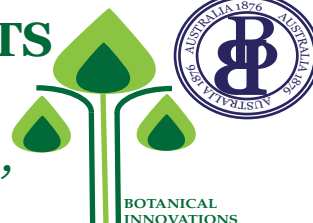


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## BOTANICAL INNOVATIONS



Botanical Innovations is an Australian Bio Technology company adding value to naturally occurring elements to create functional food and beverage solutions, which combine taste and function.

The company invests heavily in research and development leading to innovations, new products and proprietary production methodologies and the development of a unique range of **AUSTRALIAN FUNCTIONAL NUTRACEUTICAL, Flavours, Frances & Ingredients for Functional Foods & Beverages, Natural Healthcare and Cosmeceutical Applications.**

The Botanical Innovations product range includes, Plant Extracts, Fermented Plant Extracts, Cold Pressed Seed Oils, Fermented Fruit & Vinegar, Fruit Powders & Essential Oils.

By unlocking nature's secrets Botanical Innovations has created new classes of flavours, fragrances and ingredients that contain phenolic rich phyto nutrients the natural chemical compounds produced by plants from photosynthesis.

Our database of scientific literature provides our customers with access to the latest research and applications for our unique range of products.

Botanical Innovations products include:

### **Plant Extracts and Fermented Plant Extract**

Grape Seed Extracts  
Grape Skin Extracts  
Apple Peel Extracts  
French Oak Extracts

Australian Native Essential Oils and Extracts  
Buddha Wood Essential Oil  
Buddha Wood Leaf Stem Cell Extract  
Buddha Wood Oil  
Buddha Wood Aromatic Water  
Quandong Seed Oil

### **Cold Pressed Oils**

Grape Seed Oil  
Cherry and Macadamia Seed Oil  
Grape and Macadamia Seed Oil

### **Essential Oils and Aromatic Waters**

Basil  
Oregano  
Persian Catnip  
Thyme

### **Fruit Powders**

Grape Seed  
Apple Peel



**WINNER**  
INGREDIENT  
INNOVATION

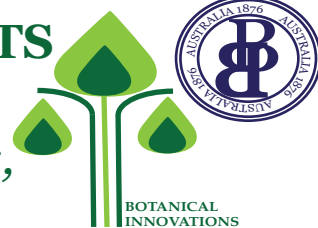


**FINALIST**



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## AUSTRALIAN COLD CLIMATE GRAPES PRODUCTS



### **Extracts**

*Red Grape Seed Extract  
White Grape Seed Extract  
Grape Seed Extract  
White Grape Skin Extract  
Red Grape Skin Extract  
Red Wine Extract Powder  
White Wine Extract Powder*

### **Fermented Extracts**

*Fermented Red Grape Seed Extract  
Fermented White Grape Seed Extract  
Fermented Red Grape Skin Extract  
Fermented White Grape Skin Extract  
Fermented Grape Extract*

### **Oils**

*Cold Pressed Grape Seed Oil*

### **Wine Powders**

*Red Wine Powder (Shiraz)  
Red Wine Powder (Cabernet Sauvignon)  
Tawny Port Powder  
Sparkling Brut Powder  
Chardonnay Powder  
Sauvignon Blanc Powder*

### **Fibre Powders**

*White Grape Fibre Powder  
Red Grape Fibre Powder*





# APPLE CIDER VINEGAR POWDER WITH 'MOTHER'



# BOTANICAL INNOVATIONS GROUP

[WWW.BOTANICALINNOVATIONS.COM.AU](http://WWW.BOTANICALINNOVATIONS.COM.AU)



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## Recent Published Scientific Studies about the health benefits of Vinegar

### Published Research Abstracts

#### Examination of the anti glycemic properties of vinegar in healthy adults.

Publication: Annals of Nutrition and Metabolism 2010: 56 (1) pp74---9

Authors: Johnston CS, Steplewska I, Long CA, Harris LN, Ryals RH

#### Background

Vinegar reduces postprandial glycemia (PPG) in healthy adults. This study investigated the vinegar dosage (10 vs. 20 g), timing (during mealtime vs. 5 h before meal) and application (acetic acid as vinegar vs. neutralized salt) for reducing PPG.

#### Methods

Four randomized crossover trials were conducted in adults (n = 9---10/trial) with type 2 diabetes (1 trial) or without diabetes (3 trials). All trials followed the same protocol: a standardized meal the evening prior to testing, an overnight fast ( 1 10 h) and 2---hour glucose testing following consumption of a bagel and juice test meal (3 trials) or dextrose solution (1 trial). For each trial, PPG was compared between treatments using area---under---the---curve calculations 120 min after the meal.

#### Results

Two teaspoons of vinegar ( 10 g) effectively reduced PPG, and this effect was most pronounced when vinegar was ingested during mealtime as compared to 5 h before the meal. Vinegar did not alter PPG when ingested with monosaccharides, suggesting that the antiglycemic action of vinegar is related to the digestion of carbohydrates. Finally, sodium acetate did not alter PPG, indicating that acetate salts lack antiglycemic properties.

#### Conclusions

The antiglycemic properties of vinegar are evident when small amounts of vinegar are ingested with meals composed of complex carbohydrates. In these situations, vinegar attenuated PPG by 20% compared to placebo

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Vinegar supplementation lowers glucose and insulin responses and increases satiety after a bread meal in healthy subjects.

Publication: European Journal of Clinical Nutrition 2005 Sept: 59 (9) pp 983---8

Authors: Ostman E1, Granfeldt Y, Persson L, Björck I.

### **Objective**

To investigate the potential of acetic acid supplementation as a means of lowering the glycaemic index (GI) of a bread meal, and to evaluate the possible dose---response effect on postprandial glycaemia, insulinaemia and satiety.

### **Subjects and Setting**

In all, 12 healthy volunteers participated and the tests were performed at Applied Nutrition and Food Chemistry, Lund University, Sweden.

### **Intervention**

Three levels of vinegar (18, 23 and 28 mmol acetic acid) were served with a portion of white wheat bread containing 50 g available carbohydrates as breakfast in randomized order after an overnight fast. Bread served without vinegar was used as a reference meal. Blood samples were taken during 120 min for analysis of glucose and insulin. Satiety was measured with a subjective rating scale.

### **Results**

A significant dose---response relation was seen at 30 min for blood glucose and serum insulin responses; the higher the acetic acid level, the lower the metabolic responses. Furthermore, the rating of satiety was directly related to the acetic acid level. Compared with the reference meal, the highest level of vinegar significantly lowered the blood glucose response at 30 and 45 min, the insulin response at 15 and 30 min as well as increased the satiety score at 30, 90 and 120 min postprandially. The low and intermediate levels of vinegar also lowered the 30 min glucose and the 15 min insulin responses significantly compared with the reference meal. When GI and II (insulinaemic indices) were calculated using the 90 min incremental area, a significant lowering was found for the highest amount of acetic acid, although the corresponding values calculated at 120 min did not differ from the reference meal.



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### Conclusion

Supplementation of a meal based on white wheat bread with vinegar reduced postprandial responses of blood glucose and insulin, and increased the subjective rating of satiety. There was an inverse dose---response relation between the level of acetic acid and glucose and insulin responses and a linear dose---response relation between acetic acid and satiety rating. The results indicate an interesting potential of fermented and pickled products containing acetic acid.

## FLAVOURS, FRAGRANCES AND INGREDIENTS

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### Anti obesogenic effect of apple cider vinegar in rats subjected to a high fat diet

Publication: Ann Cardiol Angeiol (Paris). 2016 Jun;65(3):208---13.

Authors: Bouderbala H, Kaddouri H, Kheroua O2, Saidi D

#### **Aim of the Study**

The search of new antiobesogenic treatments based on medicinal plants without or with minimal side effects is a challenge. In this context, the present study was conducted to evaluate the anti---obesogenic effect of apple cider vinegar (ACV) in Wistar rats subjected to a high fat diet.

#### **Materials and Methods**

Eighteen male Wistar rats (140±5g) were divided into 3 three equal groups. A witness group submitted to standard laboratory diet and two groups subjected to a high fat diet (cafeteria diet); one receives a daily gavage of apple cider vinegar (7mL/kg/d) for 30 days. Throughout the experiment monitoring the nutritional assessment, anthropometric and biochemical parameters is achieved.

#### **Results**

In the RCV vs RC group, we observed a highly significant decrease ( $P<0.001$ ) in body weight and food intake. On the other hand, the VCP decreases very significantly different anthropometric parameters: BMI ( $P<0.01$ ), chest circumference and abdominal circumference ( $P<0.001$ ), decreases serum glucose levels (26.83%) and improves the serum lipid profile by reducing plasma levels of total cholesterol (34.29%), TG (51.06%), LDL (59.15%), VLDL (50%) and the total lipid (45.15%), and increasing HDL (39.39%), thus offering protection against oatherogenic risk (61.62%).

#### **Conclusion**

This preliminary study indicates that the metabolic disorders caused by high fat diet (cafeteria) are thwarted by taking apple cider vinegar which proves to have a satiating effect, antihyperlipidemic and hypoglycemic effects, and seems prevent the atherogenic risk.



## FLAVOURS, FRAGRANCES AND INGREDIENTS

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Apple cider vinegar modulates serum lipid profile, erythrocyte, kidney, and liver membrane oxidative stress in ovariectomized mice fed high cholesterol.

Publication: Membrane Biology Journal 2014 Aug;247(8):667---73

Authors: Nazıroğlu M1, Güler M, Özgül C, Saydam G, Küçükayaz M, Sözbir E.

### Abstract

The purpose of this study was to investigate the potentially beneficial effects of apple cider vinegar (ACV) supplementation on serum triglycerides, total cholesterol, liver and kidney membrane lipid peroxidation, and antioxidant levels in ovariectomized (OVX) mice fed high cholesterol.

Four groups of ten female mice were treated as follows: Group I received no treatment and was used as control. Group II was OVX mice. Group III received ACV intragastrically (0.6% of feed), and group IV was OVX and was treated with ACV as described for group III.

The treatment was continued for 28 days, during which the mice were fed a high---cholesterol diet.

The lipid peroxidation levels in erythrocyte, liver and kidney, triglycerides, total, and VLDL cholesterol levels in serum were higher in the OVX group than in groups III and IV. The levels of vitamin E in liver, the kidney and erythrocyte glutathione peroxidase (GSH---Px), and erythrocyte---reduced glutathione (GSH) were decreased in group II. The GSH---Px, vitamin C, E, and  $\beta$ ---carotene, and the erythrocyte GSH and GSH---Px values were higher in kidney of groups III and IV, but in liver the vitamin E and  $\beta$ ---carotene concentrations were decreased.

In conclusion, ACV induced a protective effect against erythrocyte, kidney, and liver oxidative injury, and lowered the serum lipid levels in mice fed high cholesterol, suggesting that it possesses oxidative stress scavenging effects, inhibits lipid peroxidation, and increases the levels of antioxidant enzymes and vitamin.

## FLAVOURS, FRAGRANCES AND INGREDIENTS

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Effects of apple cider vinegars produced with different techniques on blood lipids  
in high cholesterol fed rats.

Publication: Journal of Agricultural Food Chemistry 2011 Jun 22;59(12):6638---44.  
Authors: Budak NH, Kumbul Doguc D, Savas CM, Seydim AC, Kok Tas T, Ciris MI, Guzel Seydim ZB.

### **Abstract**

Red delicious apples were used to produce natural apple cider with and without inclusion of maceration. Traditional surface and industrial submersion methods were then applied to make vinegar from apple ciders.

Apple cider vinegar samples produced with inclusion of maceration in the surface method had the highest total phenolic content, chlorogenic acid, ORAC, and TEAC levels.

Cholesterol and apple vinegar samples were administered using oral gavage to all groups of rats except the control group.

Apple cider vinegars, regardless of the production method, decreased triglyceride and VLDL levels in all groups when compared to animals on high---cholesterol diets without vinegar supplementation.

Apple cider vinegars increased total cholesterol and HDL and LDL cholesterol levels and decreased liver function tests when compared to animals on a high---cholesterol diet without vinegar supplementation. A high---cholesterol diet resulted in hepatic steatosis. VSBM and VSB groups significantly decreased steatosis.

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### Vinegar Published Research Papers

#### Functional Properties of Vinegar

Article attached

Publication: Journal of Food Science May 2014

Authors: Budak N, Akin Z, Seydum A, Greene A, Guzel---Seydum B

#### Vinegar: Medicinal Uses and Antiglycemic Effect

Article attached

Publication: MedGenMed 2006: 8(2) 61

Authors: Johnston C, Gaas C

Antimicrobial activity of apple cider vinegar against *Escherichia coli*,  
*Staphylococcus aureus* and *Candida albicans*; downregulating cytokine and  
microbial protein expression

Article attached

Publication: nature.com/scientific reports

Authors: Darshna Yagnik, Vlad Serafn & Ajit J. Shah

#### Microorganisms in Fermented Apple Beverages: Current Knowledge and Future Directions

Article attached

Publication: Microorganisms 2017, 5, 39; [www.mdpi.com/journal/microorganisms](http://www.mdpi.com/journal/microorganisms)

Authors: Fabien J. Cousin ID , Rozenn Le Guellec, Margot Schlusshuber, Marion  
Dalmasso ID , Jean-Marie Laplace and Marina Cretenet

### Appendix

#### Catalyst Video Transcript Vinegar Medicinal Benefits



# SCIENTIFIC REPORTS

OPEN

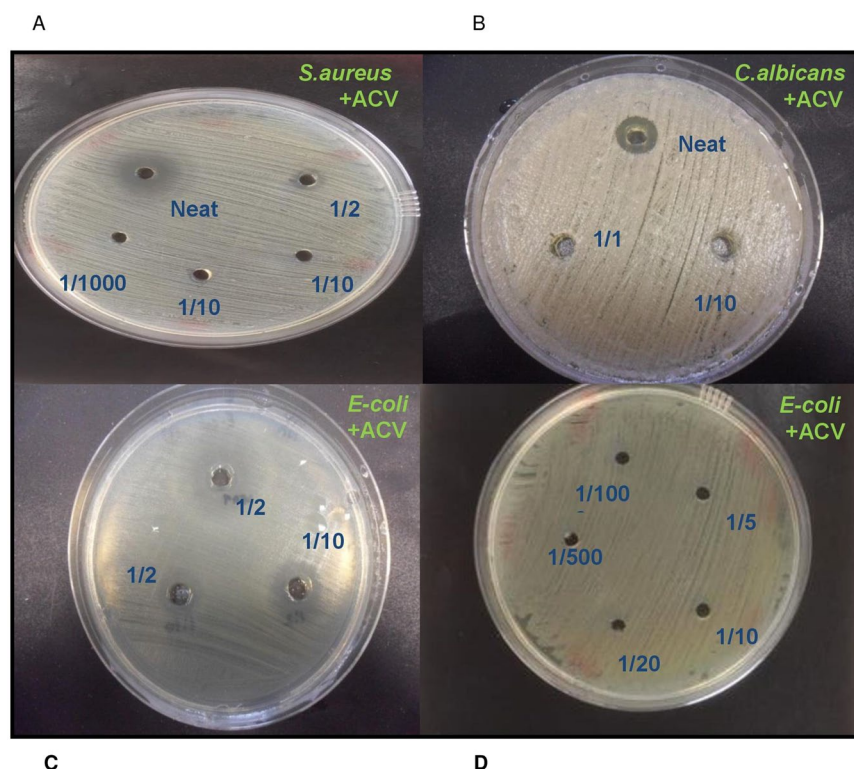
## Antimicrobial activity of apple cider vinegar against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*; downregulating cytokine and microbial protein expression

Darshna Yagnik, Vlad Serafin & Ajit J. Shah

The global escalation in antibiotic resistance cases means alternative antimicrobials are essential. The aim of this study was to investigate the antimicrobial capacity of apple cider vinegar (ACV) against *E. coli*, *S. aureus* and *C. albicans*. The minimum dilution of ACV required for growth inhibition varied for each microbial species. For *C. albicans*, a 1/2 ACV had the strongest effect, *S. aureus*, a 1/25 dilution ACV was required, whereas for *E. coli* cultures, a 1/50 ACV dilution was required ( $p < 0.05$ ). Monocyte co-culture with microbes alongside ACV resulted in dose dependent downregulation of inflammatory cytokines (TNF $\alpha$ , IL-6). Results are expressed as percentage decreases in cytokine secretion comparing ACV treated with non-ACV treated monocytes cultured with *E. coli* (TNF $\alpha$ , 99.2%; IL-6, 98%), *S. aureus* (TNF $\alpha$ , 90%; IL-6, 83%) and *C. albicans* (TNF $\alpha$ , 83.3%; IL-6, 90.1%) respectively. Proteomic analyses of microbes demonstrated that ACV impaired cell integrity, organelles and protein expression. ACV treatment resulted in an absence in expression of DNA starvation protein, citrate synthase, isocitrate and malate dehydrogenases in *E. coli*; chaperone protein DNaK and ftsZ in *S. aureus* and pyruvate kinase, 6-phosphogluconate dehydrogenase, fructose biphosphate were among the enzymes absent in *C. albicans* cultures. The results demonstrate ACV has multiple antimicrobial potential with clinical therapeutic implications.

Antibiotic resistance is rapidly becoming a major worldwide problem. There has been a steady increase in the number of pathogens that show multiple drug resistance. In fact the World Health Organization predicts that infections involving antibiotic resistant pathogens will pose major patient care management issues in the future<sup>1</sup>. This will inevitably lead to an increase in hospital stays, cost, patient morbidity and mortality. In the immunocompromised and at risk patients severe microbial infections can result in sepsis. Sepsis can rapidly lead to systemic inflammation and organ failure<sup>2</sup>. In response to microbial invasion, the innate immune system reacts by triggering tissue damage. Mononuclear cells recognize pathogens associated with molecular patterns (PAMPs) present on the microbial surface. This results in intracellular signaling cascades which initiate pro-inflammatory cytokine and chemokine release into the blood circulation. Unchecked, the chemokines will continue to recruit more immune cells to the site of infection which release further pro-inflammatory cytokines enhancing inflammation in a continuous feedback loop<sup>3</sup>. Essentially, antibiotics are antimicrobials but can also act as immune modulators reducing the release of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF $\alpha$ , IL-8, and interferon-gamma (INF-gamma). Antibiotics can also affect mononuclear phagocytic function and modulate the activity of nuclear transcription factors such as NF- $\kappa$ B and activator proteins<sup>4–7</sup>. Microorganisms such as *E. coli*, *S. aureus* and *C. albicans* form part of the human microbiota. However pathogenic forms of these microbes have been implicated in blood or urinary tract infections, gastroenteritis, endocarditis, soft tissue infections and organ malfunction<sup>8–10</sup>.

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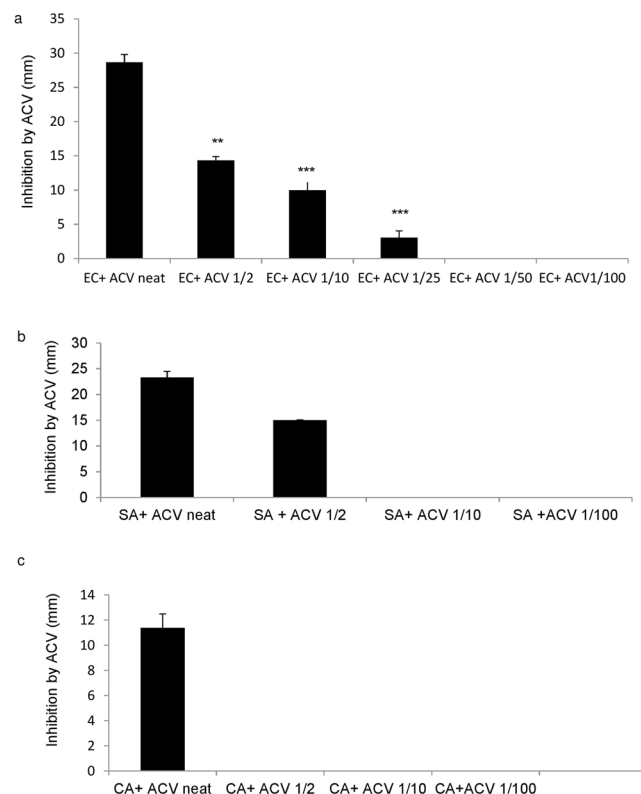
**Figure 1.** Effect of varying concentrations of ACV on microbial growth after incubation at 37 °C for 24 h. (a) *S. aureus*; (b) *C. albicans*; (c) *E. coli* (d) *E. coli*. ACV was either applied neat or diluted 1:2 or 1:10 v/v in distilled water. Zones of microbial growth inhibition are indicated by clear zones and vary with ACV dilutions for each microbe. Photographs were taken using a 20 Mega pixel Samsung camera.

The anti-microbial agents used to treat gram negative infections such as  $\beta$ -lactams, fluoroquinolones, sulfamethoxazole and trimethoprim are becoming increasingly ineffective. Strains of *S. aureus* have emerged with reduced susceptibility to vancomycin and methicillin<sup>6,11,12</sup>. Furthermore, antibiotic action itself can be problematic in terms of cell membrane permeability, intracellular inactivation and the inability to reach intracellular structures in which organisms can hide. Alternative supplementation that can combat a plethora of microbes without concurrent side effects would be of significant healthcare interest as the discovery of effective new antibiotic has been slow but should be a global priority.

The Old Testament and Hippocrates reported on the use of ACV in combination with honey to combat infection and protect open skin wounds. Historically, vinegar has been produced and sold as a commercial commodity for over 5000 years. In fact up until the sixth century BC, the Babylonians were making vinegars for consumption as well as for use in healing<sup>13</sup>. Vinegar is the resultant product when ethyl alcohol is converted to acetic acid by *Acetobacter*. It can be produced by different methods and a variety of raw materials such as wine, malted barley, alcohol, fruits and cider<sup>14</sup>. ACV is produced from cider that has undergone acetous bioconversion and has relatively low acidity (5% acetic acid). It also contains organic acids, flavonoids, polyphenols, vitamins and minerals<sup>15</sup>. ACV has been hailed as a supplement aiding weight loss, hyperlipademia, hypercholesterlaemia, nutritional support, antioxidant defence and lowering blood pressure. Utilising organic acids as nutritional supplements has been regarded as safe and can eliminate harmful intestinal bacteria<sup>16–18</sup>. The positive impact of dietary ACV supplementation has been highlighted *in vivo*. ACV decreased the serum lipid profile in mice fed a high cholesterol diet over 28 days. Intragastric ACV addition induced a protective effect against erythrocyte, kidney and liver oxidative injury as well as lowering cholesterol levels<sup>16</sup>. ACV also decreased blood triglyceride and very low density lipoprotein levels in rats which had induced cholesterol induced hepatic steatosis<sup>18</sup>. Despite the known health benefits of dietary organic acid supplementation, to the best of our knowledge the direct effect of ACV on microbes and mononuclear leucocytes has not been examined. The aim of the present study was to investigate the antimicrobial activity of ACV on microbes and associated inflammatory pathways.

## Results

**The antibacterial and antifungal activity of ACV against *E. coli*, *S. aureus* and *C. albicans*.** In order to determine the anti-microbial activity of ACV, *E. coli*, *S. aureus* and *C. albicans* were directly cultured with different concentrations of ACV. Figure 1 represents the experimental results. The minimum dose required to restrict growth for *C. albicans* was neat, undiluted ACV (5% acidity), for *S. aureus* it was a 1/2 dilution (2.5% acidity) and for *E. coli*, growth was restricted at a significantly lower dilution of 1/50 (equivalent to 0.1% acidity). We also measured the equivalent zones of inhibition (in mm) for each of the microbes at varying dilutions of ACV which is depicted in the photographs of the culture plates (Fig. 2). To translate the MIC into supplementary



**Figure 2.** Inhibition of microbe growth by ACV after incubation for 24 h at 37 °C. **(a)** *E. coli*; **(b)** *S. aureus* **(c)** *C. albicans*. Zone of inhibition was measured in mm. These experiments represent data from three repeats. EC = *E. coli*, SA = *S. aureus*, CA = *C. albicans*, ACV = Apple cider vinegar.

tablet dosages required, we tested concentration ranges from 400 µg/ml in doubling microdilutions to the lowest of 3.1 µg/ml against each microbe. The MIC for ACV tablets at which no growth was visible was 62 µg/ml for *E. coli*; 125 µg/ml for *S. aureus* and 250 µg/ml for *C. albicans* respectively. Both sets of results were also confirmed further by microdilution. We used the Braggs ACV for all future experiments at the minimum inhibitory dilution required for each organism.

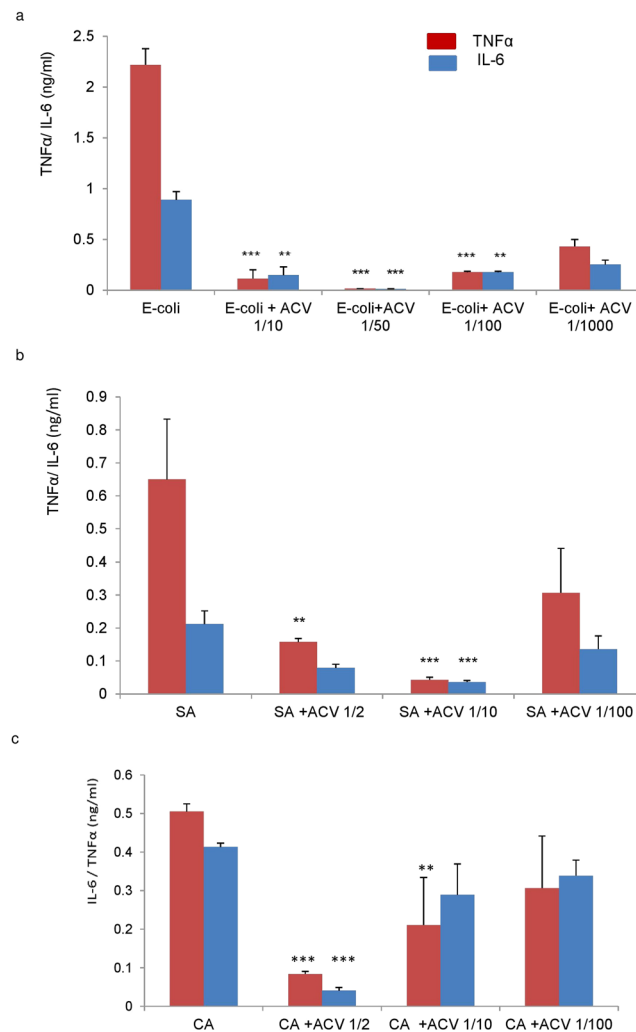
**Downregulation of pro-inflammatory cytokine secretion by ACV in monocytes exposed to microbes.** The microbes utilised in our study have been extensively studied and are known to cause inflammation through their capacity to stimulate the leucocyte pro-inflammatory cytokine cascades<sup>19,20</sup>. Hence, we proceeded to measure mononuclear derived TNF-α and IL-6 cytokines as indicators of inflammation which are also the markers of choice for clinical diagnosis of septic infections<sup>21</sup>.

Figure 3 depicts the effects of a dose dependent reduction in TNFα and IL-6 release from monocytes which have been co-cultured with ACV, together with either *C. albicans*, *E. coli* or *S. aureus* for 24 h.

Consistent with the microbial growth inhibition data depicted in Fig. 2, the effect of ACV at the minimum inhibitory concentration of 1/50 resulted in a significant reduction in monocyte derived TNFα ( $p = 0.008$ ) and IL-6 release ( $P = 0.001$ ) in monocytes cultured with *E. coli*. For *S. aureus* the minimum inhibitory concentration for ACV was found to be 1/10 in terms of reduction of TNFα ( $p = 0.011$ ) and IL-6 ( $p = 0.03$ ). For *C. albicans* the minimum inhibitory dilution was lower at 1/2 dilution, for TNFα ( $p = 0.003$ ) and IL-6 ( $p = 0.008$ ). It was imperative to ascertain whether the monocytes were alive during inoculation with the various microbes especially after incubation for 24 h at 37 °C. We added Trypan blue directly to monocytes which had been co-cultured with microbes after 2, 6 and 24 h. Light microscopy revealed that greater than 90% of cells were alive after 24 h in all co-cultures as demonstrated in (Fig. 4a,b,c) which represents the light microscopic images of monocytes and the microbes in co-cultures.

**Upregulation of phagocytic capacity.** We also investigated whether ACV could have an effect on the phagocytic function of monocytes alone and also after a 4 h exposure to microbes with or without ACV treatment. A 14.2, 13.7 and 20.4% increase in monocyte phagocytic capacity was observed after *E. coli*, *S. aureus* and *C. albicans* co-culture with ACV respectively and in comparison to the resting unstimulated monocytes. Results are expressed as the mean and SD of 3 similar experiments (Table 1). This suggests that ACV can increase phagocytic potential in monocytes which is significant as microbial phagocytosis is a key effector function of innate immunity<sup>22</sup>.





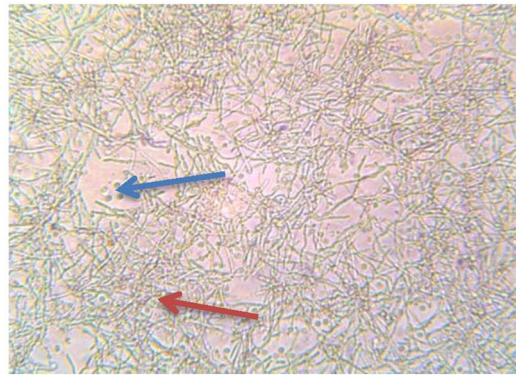
**Figure 3.** Effect of ACV on pro-inflammatory cytokine secretion from human monocytes infected with (a) *E. coli*; (b) *C. albicans* and (c) *S. aureus* after incubation for 24 h at 37 °C. ACV was added at dilutions of 1/10, 1/25, 1/50, 1/100 or 1/1000. EC = *E. coli*, SA = *S. aureus*, CA = *C. albicans*. The minimum inhibitory dilution of ACV required for significant pro-inflammatory downregulation varied with each microbe. For TNFα, a 1/50 ACV minimum inhibitory dilution was required for EC,  $p = 0.0008$ ; 1/10 for SA,  $p = 0.01$ ; 1/2 for CA,  $p = 0.0003$  respectively. For IL-6, 1/50 ACV dilution was required for EC,  $p = 0.0008$ ; 1/10 for SA,  $p = 0.03$ ; 1/2 for CA,  $p = 0.008$  respectively. Results represented are mean  $\pm$  SD of 3 experiments. We used student's paired t-tests for statistical evaluation (Excel 2017) with statistical significance taken when  $p < 0.05$ . EC = *E. coli*, SA = *S. aureus*, CA = *C. albicans*, ACV = Apple cider vinegar.

**Proteomic results of *E. coli*, *S. aureus* and *C. albicans* after exposure to ACV.** The bottom-up proteomic study of ACV treated *E. coli* cultures revealed the absence in detection of key enzymes; citrate synthase, isocitrate dehydrogenase, deoxyribose-phosphate idolase, malate dehydrogenase, aminomethyltransferase and formate acetyltransferasesuccinyl-CoA ligase (Table 2). An absence in acyl carrier protein, DNA protein including DNA protection during starvation protein, integration host factor subunit alpha and ribosome associated inhibitor A was evident. Following ACV treatment *S. aureus* cultures failed to express 50s ribosomal proteins L2, L15, L23, L24, enzymes; alcohol dehydrogenase, catalase, formate acetyltransferase, L-lactate dehydrogenase-2, ornithine aminotransferase and serine hydroxymethyl transferase (Table 3). Cell division protein ftsZ and chaperone protein Dnak were also not detected. However an important pentose phosphate pathway enzyme: 6 phosphogluconate dehydrogenase decarboxylating was displayed. Table 4 demonstrates that key enzymes required for glycolysis and candida immunogenicity were undetected after 24 h of exposure to ACV in *C. albicans*. These incorporated fructose bisphosphate aldolase, phosphogluconate dehydrogenase, pyruvate kinase and peptidyl-propyl cis-trans isomerase.

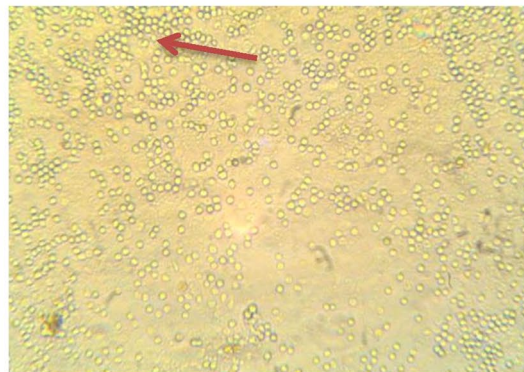
## Discussion

ACV has multiple antimicrobial properties on different microbial species, affecting microbe growth, suppressing mononuclear cytokine and phagocytic responses. The tandem mass spectroscopy results are in cohesion with

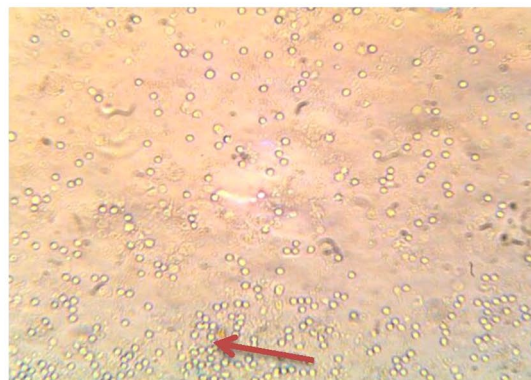
a



b



c



**Figure 4.** (a–c) Photos of monocytes in co-culture with microbes. Monocytes were cultured with the microbes and ACV. Trypan blue addition indicated over 95% viability. Red arrows indicate microbes and the blue arrow shows monocytes in Fig. 4a which are not visible in Fig. 4b and c since they have been covered by the microbes. Photographs were taken after 24 h incubation at 37 °C under  $\times 100$  magnification using a light microscope indicated over 90% viability with monocytes at 2, 4, 6 and 24 h of co-culture (24 h photos shown). A = *C. albicans*, B = *E. coli* and C = *S. aureus* respectively.

these observations. The microbes underwent significant impairment following ACV addition which damaged cell integrity, structural and metabolic proteins as well as nuclear material. Indeed the enzymes citrate synthase, isocitrate dehydrogenase, malate dehydrogenase, aminomethyltransferase and formate acetyltransferasesuccinyl-CoA ligase are crucial for *E. coli* growth, gene regulation and central, intracellular carbon metabolism. An absence of these enzymes would affect glycolytic, tricarboxylic acid cycle, pentose phosphate, glyoxylate shunt and oxidative phosphorylation pathways in *E. coli*<sup>23</sup>. Furthermore, the absence of DNA binding protein from starved cells, is significant as it protects *E. coli* and functions to control gene regulation during cell starvation<sup>24</sup>. With respect to other proteins, we observed the presence of ribosomal proteins (L1, L6 and 30 s ribosomal proteins (S1, S4, S6,

Monocyte co-culture conditions	Monocyte phagocytic capacity expressed as % change increase in side scatter. (mean $\pm$ SD)
Medium	6.1 $\pm$ 1.4
<i>E. coli</i>	9.0 $\pm$ 2.4
<i>S. aureus</i>	22.3 $\pm$ 2.2
<i>C. albicans</i>	31.9 $\pm$ 2.4
<i>E. coli</i> + ACV	23.2 $\pm$ 3.7
<i>S. aureus</i> + ACV	36.0 $\pm$ 5.2
<i>C. albicans</i> + ACV	52.3 $\pm$ 4.1

**Table 1.** Effect of ACV on human monocyte phagocytic capacity. *In vitro* differentiated monocytes were incubated with microbes for 4 h at 37 °C. Cells cultured with microbes with or without ACV were washed and processed for detection on the Beckton Dickinson flow cytometer. An analysis of changes in regional gated profiles and % shift in side scatter was measured. Data is presented as mean  $\pm$  SD of 3 similar experiments.

Protein Name	Mass (Da)	Control <i>E. coli</i> culture	ACV Treated <i>E. coli</i> culture
30S ribosomal protein S1	—		*
30S ribosomal protein S11	13950		*
30S ribosomal protein S4	23512		*
30S ribosomal protein S6	15177		*
30S ribosomal protein S7	17593	*	
30S ribosomal protein S8	14175	*	
50S ribosomal protein	14923	*	*
50S ribosomal protein L1	24714		*
50S ribosomal protein L17	14413	*	*
50S ribosomal protein L2	29956	*	
50S ribosomal protein L6	18949		*
60 kDa chaperonin	57464	*	*
Acyl carrier protein	8693	*	
Aminomethyltransferase	40235	*	
Autonomous glycyl radical cofactor	—		*
Citrate synthase	48383	*	
Cytidine deaminase	31805	*	
Deoxyribose-phosphate aldolase	27958	*	
DNA protection during starvation protein	18684	*	
DNA-binding protein H-NS	15587		*
DNA-binding protein HU-alpha	9529	*	
DNA-binding protein HU-beta	9220	*	
Elongation factor Tu 1	43427	*	*
Enolase	45683	*	*
Formate acetyltransferase 1	85588	*	
Glutamate/aspartate periplasmic-binding protein	33513	*	
Glyceraldehyde-3-phosphate dehydrogenase	35681	*	*
Integration host factor subunit alpha	11347	*	
Isocitrate dehydrogenase [NADP]	46070	*	
Major outer membrane lipoprotein Lpp	8375		*
Malate dehydrogenase	32488	*	
Outer membrane protein A	37292	*	*
Ribosome-associated inhibitor A	12777	*	
Succinate dehydrogenase flavoprotein subunit	65008	*	*
Succinyl-CoA ligase [ADP-forming] subunit beta	42244	*	
Transaldolase B	35368		*
Uridine phosphorylase	27313	*	*

**Table 2.** List of *E. coli* proteins identified following ACV treatment. *E. coli* were cultured with 1/50 dilution of ACV or alone in broth for 24 hours at 37 °C in a shaking incubator. After which mass spectroscopy analysis was carried out. The “\*” indicates the presence of protein whilst the blank region denotes no detection of that particular protein.



Protein name	Mass (Da)	Control <i>S. aureus</i> culture	ACV-Treated <i>S. aureus</i> culture
30S ribosomal protein S1	43250	*	*
30S ribosomal protein S12	15334	*	
30S ribosomal protein S2	29133	*	
30S ribosomal protein S3	24085	*	*
30S ribosomal protein S4	22999	*	
30S ribosomal protein S5	17732	*	
30S ribosomal protein S6	11588	*	
30S ribosomal protein S7	17783	*	*
30S ribosomal protein S8	14822	*	
50S ribosomal protein L1	24693	*	
50S ribosomal protein L13	16323	*	*
50S ribosomal protein L14	13241	*	*
50S ribosomal protein L15	15587	*	
50S ribosomal protein L2	30194	*	
50S ribosomal protein L21	11326	*	*
50S ribosomal protein L23	10599	*	
50S ribosomal protein L24	11529	*	
50S ribosomal protein L25	23773	*	*
50S ribosomal protein L29	8085		*
50S ribosomal protein L4	22451	*	*
50S ribosomal protein L6	19774		*
6-phosphogluconate dehydrogenase, decarboxylating	51941		*
Alcohol dehydrogenase	36424	*	
Arginine deiminase	47113	*	*
Bacterial non-heme ferritin	23773	*	*
Catalase	58457	*	
Cell division protein FtsZ	41012	*	
Chaperone protein DnaK	66338	*	
DNA-binding protein HU	9620	*	*
Elongation factor Tu	43134	*	*
Enolase	47145	*	*
ESAT-6 secretion system extracellular protein A	11029	*	
Formate acetyltransferase	85264	*	
Fructose-bisphosphate aldolase class I	32907		*
Isocitrate dehydrogenase [NADP]	46451		*
L-lactate dehydrogenase 2	34468	*	
Ornithine aminotransferase 2	43675	*	
Ornithine carbamoyltransferase, catabolic	37853	*	*
Probable malate:quinone oxidoreductase	56135	*	*
Putative universal stress protein SA1532	18521	*	*
Pyruvate dehydrogenase E1 component subunit beta	35194	*	*
Serine hydroxymethyltransferase	45384	*	

**Table 3.** List of *S. aureus* proteins identified following ACV treatment. *S. aureus* were cultured with 1/10 dilution of ACV or alone in broth for 24 hours at 37 °C in a shaking incubator. After which mass spectroscopy analysis was carried out. The “\*” indicates the presence of protein whilst the blank region denotes no detection of that particular protein.

S7, S8, S11) in ACV treated *E. coli* compared to control *E. coli*. These are mostly RNA binding proteins hence their presence could have been due to partial disintegration of 50s and 30s ribosomal breakdown whereas these subunits might remain intact in untreated *E. coli* cultures. The absence of ribosome associated inhibitor A could

Protein name	Mass (Da)	Control <i>C. albicans</i> culture	ACV- Treated <i>C. albicans</i> culture
40S ribosomal protein S1	29083	*	*
6-phosphogluconate dehydrogenase	71270	*	
Alcohol dehydrogenase 1	37255	*	*
Elongation factor 1-alpha 1	50436	*	*
Elongation factor 2	93865	*	*
Enolase 1	47202	*	*
Fructose-bisphosphate aldolase	39362	*	
Glucose-6-phosphate isomerase	61148		*
Glyceraldehyde-3-phosphate dehydrogenase	35925	*	*
Heat shock protein SSA1	70452	*	*
Mitochondrial outer membrane protein porin	29748		*
Peptidyl-prolyl cis-trans isomerase	17678	*	
Phosphoglycerate kinase	45266	*	*
Phosphoglycerate mutase	27437	*	*
Plasma membrane ATPase 1	98083	*	*
Pyruvate decarboxylase	62744	*	*
Pyruvate kinase	55752	*	
Small heat shock protein 21	21482	*	*
Triosephosphate isomerase	26880	*	*
White colony protein WHS11	6991	*	

**Table 4.** List of *C. albicans* proteins identified following ACV treatment. *C. albicans* were cultured with 1/2 dilution of ACV or alone in broth for 24 hours at 37 °C in a shaking incubator. After which mass spectroscopy analysis was carried out. The “\*” indicates the presence of protein whilst the blank region denotes no detection of that particular protein.

interrupt *E. coli* growth cycles as it serves to minimise translational errors<sup>25</sup>. Collectively these results support the cytotoxic effects of ACV we observed on *E. coli*. ACV treated *S. aureus* cultures did not express the chaperone protein DnaK and the cell division protein ftsZ. This is significant as previous studies have shown that a non-functional DnaK system can cause a reduced tolerance to heat, oxidative, antibiotic stresses and lowered carotenoid production<sup>26</sup>. There was also an absence of gateway enzymes involved in multiple pathways such as ornithine aminotransferase<sup>27</sup>. 6-phosphogluconate dehydrogenase decarboxylating was expressed which is not surprising as it plays a critical role in protecting cells from oxidative stress<sup>28</sup>. The effect of ACV on *C. albicans* protein moieties was less dramatic, nevertheless we did detect the absence of key enzymes which are fundamental in maintaining cell integrity and biosynthetic pathways<sup>29</sup>.

There could be a strong possibility that ACV acts like other anti-pathogenic compounds in diverting monocyte responses through toll receptor signalling pathways. There is evidence that *E. coli* in particular can induce a typical M1 monocyte profile through mechanisms involving NF- $\kappa$ B activation. This results in upregulation of inflammatory cytokines TNF- $\alpha$  and PI3 kinase stimulation<sup>30</sup>. The *in vitro* M1 monocyte phenotype is also prominent in severe sepsis. This was shown in a study using baboons where a substantial mortality rate correlated with high serum levels of TNF $\alpha$  and IL-6 following induced sepsis infection<sup>31</sup>. Unchecked high levels of circulating M1 cytokines can rapidly lead to cardiac arrest and death hence any factor capable of lowering pro-inflammatory cytokine concentrations is essential in therapy<sup>32</sup>. It has been reported that ACV consists of acetic acid, flavonoids such as gallic acid, tyrosol catechin, epicatechin, benzoic acid, vanillin, caftaric acid, coumaric acid, caffeic acid, acid and ferulic acid. These constituents have been reported to affect immune defence and oxidative responses<sup>18,33</sup>.

Furthermore, the mechanism of ACV activity could be attributed in part to the apple polyphenol content. Yang *et al.* (2010) reported on the cellular protective effects of apple polyphenols on induced liver damage whereby histopathological tissue destruction was limited and liver activity maintained in mice that received the polyphenols<sup>34</sup>. The mechanisms involved were free radical scavenger action, lipid peroxidation modulation and the antioxidant upregulation capacity of ACV. Interestingly, a study by Denis *et al.* demonstrated the anti-inflammatory potential of apple phenols on gastrointestinal cell inflammation which involved downregulation of TNF and IL-6 cytokines<sup>35</sup>. Another means of action could involve the acetic acid component of ACV which is able to reduce the cell hydrogen potential hence could potentially facilitate diffusion across the plasma membrane of microbes. Furthermore, there is evidence that organic acids can alter immune responses by binding to GPR3, a G protein coupled receptor which is mostly expressed on inflammatory leukocytes<sup>36</sup>. Also, an investigation reported on upregulated blood and plasma antioxidant enzyme release after apple consumption which would encourage immune protection<sup>16</sup>.

The positive benefits of dietary ACV supplementation have been highlighted *in vivo*. ACV decreased the serum lipid profile in mice fed a high cholesterol diet over 28 days. Intragastric ACV addition induced a protective effect against erythrocyte, kidney and liver oxidative injury as well as lowering cholesterol levels<sup>18</sup>. ACV

also decreased blood triglyceride and very low density lipoprotein levels in rats which had induced cholesterol induced hepatic steatosis<sup>33</sup>. In an infection induced model of denture stomatitis, ACV addition resulted in anti-fungal activity against *Candida* Spp which was comparable to nystatin in terms of reducing microbial adherence and destruction<sup>37</sup>.

Severe infections, autoimmunity or transplantation can inevitably lead to ineffective immunity in patients. An analysis of macrophages from Crohn's disease patients revealed that they had defective responses to *E. coli* due to Crohn's related systemic immunosuppression<sup>38</sup>. A recent report showed that co-administration of ACV with *L. casei* boosted systemic and mucosal immune responses, antioxidant enzyme and growth genes in fish<sup>39</sup>. Equally, perhaps additive dietary supplementation with ACV could be of benefit in acute infections, autoimmune induced immune dysregulation or antibiotic redundancy in humans. Future studies would establish whether ACV could be used as a potential therapeutic, *In vivo* models of infection could be induced by infusing microbes systemically into mice followed by treatment with or without intraperitoneal ACV. Intragastric ACV has been fed to animals used as models of obesity and infection in the past<sup>18,37,39</sup>. ACV efficacy could be evaluated by measuring microbial burden, serum cytokine levels, leukocyte counts and tissue pathology. Side effects could include acid reflux, nausea or delayed digestion as ACV has a pH of 4.2. However, the acidity could be neutralised by the addition of sodium bicarbonate to preparations. The results of this study could have clinical implications as ACV could be used as an additive component of an antimicrobial therapeutic regimen especially in immunocompromised patients presenting with infections of the aforementioned microbes.

We conclude that ACV can have multiple antimicrobial effects directly on *E. coli*, *S. aureus* and *C. albicans*. ACV addition can also decrease induced inflammatory cytokine release during mononuclear leukocyte infection and increases monocyte phagocytic capacity. Mechanisms include alteration of the microbial protein physiology destroying structural pathogenic proteins and metabolic enzymes. Collectively our results highlight the potent antimicrobial and therefore beneficial actions of ACV. This preliminary study encourages further work on dietary ACV supplementation investigating its antimicrobial role and the constituents which could be responsible for this activity.

## Materials and Methods

**Chemical reagents, microorganisms, media and culture conditions.** A selection of microbial specimens which represented a typical gram positive, a gram negative and a yeast species were chosen for initial investigation. Microbial strains: *E. coli* strain NCTC 10418 and *S. aureus* strains NCTC 6571 were purchased from Health Protection Agency (Colindale, U.K.). *C. albicans* strain 90828 was purchased from the American Type Culture Collection (LGC Promochem).

**Reagents.** Dulbecco's modified media, dimethyl-ethyl-sulphonyl-oxide, HANKS balanced salt solution, histopaque, ethanol, phosphate buffered saline, paraformaldehyde, acetone, dithiothreitol, iodoacetamide, trypsin from porcine pancreas of proteomics grade, formic acid, acetonitrile, HPLC-grade water, methanol and Whatman Mini-UniPrep syringeless filter devices (pore size 0.45 µm) were purchased from Sigma Aldrich (Poole, U.K.). TNF-alpha, interleukin-6 (IL-6) enzyme linked immunosorbent assays (ELISAs) were purchased from Research and Development Systems (Abingdon, U.K.). Mueller hinton agar was purchased from Oxoid, UK. Braag's Apple Cider Vinegar and apple cider vinegar tablets (500 mg, Troo healthcare) were purchased from commercial sources.

**Inoculum preparation and measurement of anti-microbial activity of ACV.** Cultures of *E. coli* and *S. aureus* were grown in nutrient media whereas *C. albicans* was grown in Sabourand media. All cultures were cultivated in a shaking incubator at 37 °C for 24 h overnight prior to use. Mueller hinton agar (MHA) was prepared by dissolving 38 g in 1 litre of distilled water, boiling the mixture for 1 min, after cooling and autoclaving, the media was poured into petri dishes. The plates were left to dry and subsequently stored at 37 °C. All microbial cultures were adjusted to 0.5 McFarland's standard  $1.5 \times 10^8$  CFU/ml and  $4 \times 10^6$  CFU/ml of each organism used in experiments. Each microbe was swabbed evenly onto plates containing MHA. For sample addition, 100 µL of ACV at varying concentrations was added to the wells which were punched into the agar. The plates were then incubated at 37 °C for 24 h. Zones of inhibition surrounding samples were identified, photographed and measured in mm<sup>40</sup>. Experiments were repeated at least 5 times.

**Ethical Approval and Informed Consent.** All experimental protocols were approved by the Middlesex University Natural Sciences Ethics Committee number 2323. Further the methods were carried out in accordance to the relevant guidelines and regulations. Informed consent was received when applicable.

**Human mononuclear cell isolation procedure from whole peripheral blood.** Human leucocyte rich cones and serum were obtained from volunteer donors collected from NHS Cord blood and transplant bank at Colindale, London and treated as described previously<sup>41</sup>. Briefly the cones were washed with phosphate buffer saline to harvest the leucocyte rich cells. These were then spun on histopaque density gradient at 1200 RPM for 20 min. Monocytes were purified using the CD14 positive mononuclear portion which was isolated according to manufacturer's instruction provided with the pan monocyte isolation kit. Cells washed with HANKS balanced solution, counted and cultured into 24 well plates at  $4 \times 10^5$  cells per mL. Cells were allowed to adhere for an hour at after which non-adherent cells were washed away and full media replenished with Dulbecco's media containing 10% human serum. Monocytes were determined using light microscopy and flow cytometry phenotypic analysis of differentiation markers as described previously using CD14<sup>41</sup>. Freshly isolated monocytes were cultured with varying concentrations of ACV and either *C. albicans*, *E. coli* or *S. aureus* at counts of  $4 \times 10^6$  CFU/ml respectively



for 24 h at 37 °C and 5% CO<sub>2</sub> after which supernatants were collected and analysed for TNF- $\alpha$  or IL-6 secretion using ELISA kits following manufacturer's protocols.

**Monocyte phagocytic capacity measurement by flow cytometry.** Isolated human mononuclear cells were cultured at  $4 \times 10^5$ /mL in 24 well plates over a period of two days after which they were incubated with microbes ( $4 \times 10^6$  CFU/ml) for 4 h at 37 °C and 5 CO<sub>2</sub>. Cells were then scraped replenished in ice cold PBS containing 1 mM EDTA, washed and removed from plates. The resultant pellets were fixed in 400  $\mu$ L of 4% paraformaldehyde and analysed using a FACS Calibur flow cytometer (Beckton Dickinson Immunocytometry Systems, UK and *Cell Quest* software).

**Preparation of microbial tryptic digests for mass spectroscopy analysis.** An aliquot of microbial suspension was collected and treated over 24 h with 1/100 ACV, washed with PBS, resuspended in PBS and then treated with 1 mL of ice-cold acetone. The bacterial cells were harvested after centrifugation 13,000 g for 5 min. The pellet was dried and then reconstituted in 50 mM ammonium bicarbonate. Cells were lysed using a Soniprep 150 Plus (MSE, U.K.) for 10 s with the amplitude set at 13. Subsequently proteins were denatured and reduced with 3  $\mu$ L of 100 mM dithiothreitol in 50 mM ammonium bicarbonate at 95 °C for 5 min followed by alkylation with 6  $\mu$ L of 100 mM iodoacetamide in the dark at room temperature for 20 min. Proteins were then digested with 2  $\mu$ L of trypsin (0.1  $\mu$ g/ $\mu$ L dissolved in 50 mM ammonium bicarbonate) at 37 °C for 3 h. A further 2  $\mu$ L of trypsin was added to the sample and the mixture was incubated at 37 °C for an additional 2 h. The samples were diluted in 150  $\mu$ L 50 mM ammonium bicarbonate and passed through Mini-Uni Prep filter devices.

**Liquid Chromatography-Electrospray Ionisation Tandem Mass Spectrometry.** The tryptic peptides were analysed using a Shimadzu Prominence HPLC system hyphenated to an electrospray ionisation hybrid ion-trap time-of-flight (IT-TOF) mass spectrometer (Shimadzu, U.K.) operated in tandem mass spectrometry mode. Peptides were separated using an Ascentis Express 150  $\times$  2.1 mm, 2.7  $\mu$ m C18 column (Sigma-Aldrich, Poole, U.K.) using a flow rate of 0.21 mL/min. The column oven temperature was set to 40 °C. Data was acquired and processed using LabSolutions® software (version 3.50.348, Shimadzu, UK). A linear gradient elution profile composed of 'A' – 0.1% formic acid in water and 'B' – 0.1% formic acid in acetonitrile was used. The gradient profile was 0–40% B, 70 min; 40–90% B, 1 min; maintained at 90% B, for 3 min; 90–0% B, 1 min; and 15 min re-equilibration at 0% B. An injection volume of 40  $\mu$ L was used. Samples were kept in the auto sampler set to 4 °C. Mass spectrometry analysis was performed in MS/MS mode using positive ions electrospray. The precursors' acquisition range was set to 400–1,800  $m/z$  while the fragments acquisition range was set to 200–1,500  $m/z$ . For both precursors and fragments the ion accumulation time was set to 30 msec. The other instrument conditions were set as follows: detector voltage 1.6 kV, CID energy 70%, nebulising gas flow 1.5 L/min, CDL temperature 200 °C, heat block temperature 200 °C, interface voltage 4.5 V, detector voltage 2 kV. The data acquisition was performed in a 37.5 min interval.

**LC-MS/MS Data Processing.** MS/MS data were extracted from the resulting instrument files using Mascot Distiller software (version 2.5.1.0, Matrix Science, London, UK). For precursors peak picking the following parameters were used: correlation threshold – 0.7, minimum signal to noise ratio – 5, minimum peak  $m/z$  – 50, maximum peak  $m/z$  – 100,000, minimum peak width – 0.02 Da, expected peak width – 0.2 Da, maximum peak width – 2 Da. The MS/MS ion list was searched using Mascot search engine against all entries in Swiss-Prot database (2016\_2). For database search the following parameters were used: two missed cleavages, carbamidomethylation of cysteine (as fixed modification) and oxidation of methionine (as variable modification). The tolerance for precursor peptides was set to 10 ppm and for fragments to 0.3 Da. Peptide charges used for peak picking was +2, +3 and +4.

**Statistical analysis.** All experimental results are expressed as the mean  $\pm$  standard deviation (SD). Statistical analyses was carried out using one way ANOVA or students t-test, outcomes were considered significant where  $p < 0.05$  (when comparing apple cider vinegar treated microbes to the untreated groups in all experiments). All experiments were repeated at least 3–5 times. Analysis was carried out using Excel software version 2016.

**Data availability.** The datasets generated and analyzed during the current study reside with the corresponding author and can be made available upon request.

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## Author Contributions

D.Y. conceived and performed the experiments, data analysis and wrote the manuscript. V.S. contributed to the mass spectroscopy experiments and data analysis. A.S. contributed to manuscript writing and data review.

## Additional Information

**Competing Interests:** The authors declare that they have no competing interests.

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Review

# Microorganisms in Fermented Apple Beverages: Current Knowledge and Future Directions

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**Abstract:** Production of fermented apple beverages is spread all around the world with specificities in each country. ‘French ciders’ refer to fermented apple juice mainly produced in the northwest of France and often associated with short periods of consumption. Research articles on this kind of product are scarce compared to wine, especially on phenomena associated with microbial activities. The wine fermentation microbiome and its dynamics, organoleptic improvement for healthy and pleasant products and development of starters are now widely studied. Even if both beverages seem close in terms of microbiome and process (with both alcoholic and malolactic fermentations), the inherent properties of the raw materials and different production and environmental parameters make research on the specificities of apple fermentation beverages worthwhile. This review summarizes current knowledge on the cider microbial ecosystem, associated activities and the influence of process parameters. In addition, available data on cider quality and safety is reviewed. Finally, we focus on the future role of lactic acid bacteria and yeasts in the development of even better or new beverages made from apples.

**Keywords:** apple; cider; lactic acid bacteria; fermentation; organoleptic quality; safety improvement; microbial diversity

## 1. Introduction

Although styles of cider are extremely diverse and not easy to categorize, depending on the type of apple juices used and the degrees of sweetness, from extra dry to sweet, and alcohol content, ranging from 1.2–8% (*v/v*), cider can be defined as a fermented alcoholic beverage made from apple juice [1]. Cider production is encountered in more than 25 countries around the world in temperate regions where apple trees can flourish. The highest production is in Europe where the term cider refers strictly to fermented products [2,3]. Within Europe, the main cider-producing countries are England, Spain, France, Germany and Ireland, while smaller productions are found in Finland, Poland, Austria and Switzerland. The consumption of cider remains mainly European, accounting in 2016 for about 60% of world consumption compared with only 12% in North America [4]. There are several cider types, and traditional cider countries like Great Britain and France have their own specialties [5]. French cider tends to be sweeter than the sharper, drier cider of Great Britain, which has an alcohol content up to 8.5% (*v/v*). The fruity characteristics and aromas of French cider often are the result of ‘defecation’, in which pectins and other substances are separated from the juice. Then, the clear juice is raked off and fermented slowly and not to complete dryness [6]. In North America and Australia, the word ‘cider’ refers to the raw pressed unfermented apple juice, while ‘hard cider’ denotes a fermented product [7].

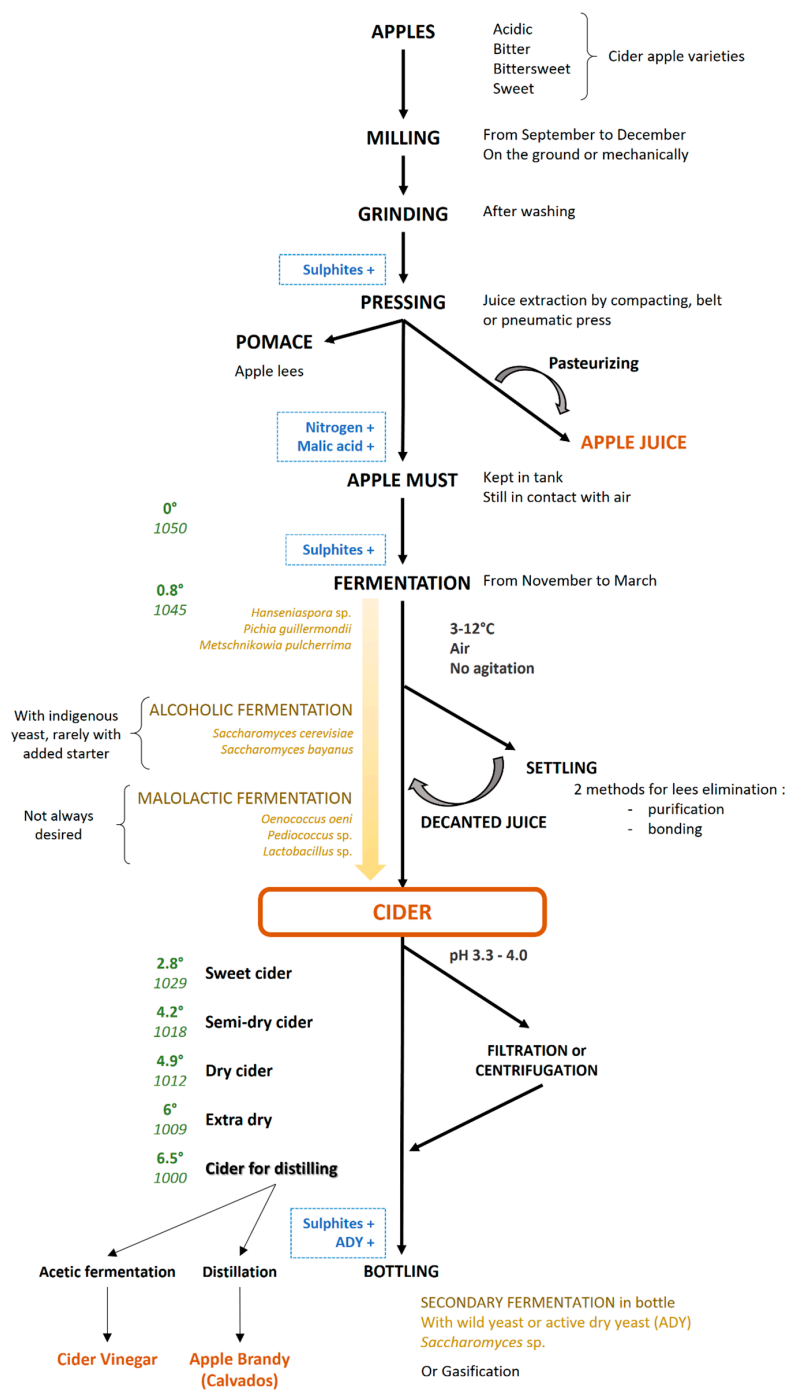
Cider is one of the oldest known beverages with a long and fascinating history. Historians broadly agree that apple trees existed along the Nile River Delta as early as 1300 BC [8], and a number of written documents citing alcoholic beverages made from apple and pear date back to ancient times, notably from Pliny, St. Augustin and Palladius [9]. By the beginning of the ninth century, cider drinking was well established in Europe, and a reference made by Charlemagne clearly confirms its popularity [10]. After the Norman Conquest of 1066, cider consumption became widespread in England, and orchards were established specifically to produce cider apples. In the first half of the twentieth century, cider was the second most consumed drink in France, behind wine, but ahead of beer [11]. Unfortunately, the damage caused to the Norman orchards during World War II together with the lack of public support resulted in a drastically reduced production, marking the decline of cider consumption in France. The current methods of cider production (quality of equipment, control assemblies and processes, stability, hygiene, neutralization of microorganisms, bottling, etc.) limit defects in the final product and make it possible to meet consumer requirements. Current cider producers use high quality standards, and ciders are elaborated under controlled conditions throughout the process.

In brief, the cider-making process (Figure 1) typically involves three main stages: apple crushing and pressing out the juice, followed by the most important stages of elaboration, fermentation. This includes classical alcoholic fermentation of sugars into ethanol performed by yeast strains and malolactic fermentation (MLF) processed by lactic acid bacteria (LAB) that can occur during the maturation. Although external sources of microorganisms may be added to the must in French traditional cider-making, alcoholic and malolactic fermentations are mainly performed by indigenous flora present on apples, on production equipment and in the cellar. Spontaneous fermentation begins within a few hours if the temperature of the must rises above 10 °C. This process is usually slow requiring at least 2–3 weeks for the main fermentation and several months for the maturation. Maturation takes place in wooden, polyester or stainless-steel casks at a controlled temperature of 3 °C–12 °C. The entire process can take from 1–6 months depending on the country. First, during alcoholic fermentation, sugars are converted mainly into ethanol and carbon dioxide by yeasts (mainly *Saccharomyces* sp.). The varietal choice and maturity of the fruits influence the sugar content of the starting must and, thus, the final ethanol level. Then, the malolactic fermentation involves the conversion of malic acid into lactic acid and carbon dioxide. Finally, the cider is bottled when its density is between 1009 and 1029 depending on the degree of sweetness desired (in France, typically extra dry, dry, half-dry and sweet). Active dry yeast (ADY) may be added in cider before bottling to obtain a naturally-carbonated beverage. The amount of residual sugar in the cider is essentially a consequence of the technological choice of the main alcoholic fermentation stoppage density and of the cider density at bottling. As shown in Figure 1, sulfites may be added at different stages of the process. Before fermentation, sulfites are added to control the natural microflora and to minimize oxidation of apple juice constituents. At bottling, sulfites are used to prevent oxidative changes and to inhibit secondary contamination [12]. After pressing, malic acid may also be added; this practice is a simple and effective way to change the acidity of the must.

Cider is a fermented beverage for which the recognition of ‘territoriality’ is important for its appreciation. The sensory profile of cider is significantly associated with microbial activities, and indigenous microorganisms may actively contribute to the expression of cider typicity. The microbial ecology of ciders is complex and includes several genera, species and strains of yeasts and bacteria [13,14]. During must production, fermentation and in the post-fermentative stage, apple juice or cider is susceptible to alteration by oxygen, enzymes, heat and/or microorganisms that can lead to a loss of nutritional and organoleptic qualities. With the increasing demand of consumers for nutritious, healthy and fresh-looking products with high organoleptic qualities, measures have been developed to prevent such alterations and to control the organoleptic characteristics of the product.

This review aims at describing the role of microbial flora in the fermentation of apple juices, highlighting the links between ecological factors, yeasts and LAB diversities and the organoleptic

properties of ciders. To date, even if ciders are safe products, research has focused mainly on the quality and safety of ciders through studies on the limitation of the development of spoilage and pathogenic microorganisms. This review will focus more on microbial quality referring to the overall effects of microbial activity, including growth, enzymatic activity and metabolic byproducts. Finally, a review of microbial diversity and the microbial contribution to the quality and safety of ciders will give us the opportunity to propose new perspectives for research on apple fermented beverages, especially through LAB activities.



**Figure 1.** Cider-making process in France. Legend: +, optional addition; alcohol degree; density; ADY: active dry yeast.

## 2. Microbial Diversity: From Apple to Cider

Regarding the microbial ecosystem, next generation sequencing strategies bring many new whole ecosystem pictures, especially regarding non-cultivable bacteria [15,16]. Such studies are very rare in the cider research area, with only one paper revealing the microbiota of wine and organic apple cider submerged vinegar production [17]. Another study described the yeast biodiversity in must and during alcoholic fermentation [18]. There is a major lack of data on cider microbiota and its dynamics during the process. Current microbial research has moved into the genomic era with increasing amounts of data available, along with decreasing costs for sequencing, especially for LAB [19] and more specifically lactobacilli [20]. Better knowledge could be easily obtained by specific (meta-)genomic analysis of cider microbiomes.

### 2.1. Yeast and Mold Diversity

Fungi (yeasts and molds) are naturally present on apples and can be found at each step of cider production. The presence of yeasts at the early stage of flower blossom has been described in various plants [21,22]. In flower nectar, yeast levels can reach densities up to  $4 \times 10^8$  cells/mL, and their frequency and abundance are directly correlated with the proportion of floral visits by bumble-bees, which thus appear as potential transmission vectors of yeasts from one flower to another in an orchard [21]. However, it seems that for the majority of plant nectars, the diversity of yeast communities is rather low [22]. On apple blossoms, yeasts have been isolated from both stigma and hypanthium surfaces, at frequencies similar to or greater than bacteria, particularly in hypanthia [23].

The apple surface is also a natural reservoir of fungi. In freshly-cut apples, fungi levels can range from 3.6–7.1 log CFU/g [24]. The dominant species identified in these cut apples were *Candida sake* and *Pichia fermentans*. Some of the fungi species on apples can be phytopathogenic species mainly included in the class Dothideomycetes, with about 95% of these in the order Capnodiales that causes damaging blemishes on apples [25,26]. A PCR-DGGE based-study of the microbiota of five varieties of Asturian apples used for the production of PDO (Protected Designation of Origin) ciders in Spain also identified *Exobasidium* sp., responsible for galls and leaf malformations, and *Mycosphaerellaceae* and *Dissociaceae* families, which produce sooty blotch and flyspeck on apples [27]. In this work, little variation in microbial diversity was found amongst the five apple varieties studied, without identifying the usual species associated with spontaneous fermentation. The authors conclude that the surface microbiota of the apples does not seem to be a determinant in the subsequent fermentation process. In contrast, another study showed that apples themselves can be the source of yeasts of technological interest [28]. This was the case with *Saccharomyces cerevisiae* yeasts, which could be found in high numbers on apples used for traditional Irish cider fermentations. In the same way, *Hanseniaspora* and *Brettanomyces/Dekkera* in ciders could be tracked back to the fruits.

The main yeasts found in cider are *Saccharomyces* yeasts. A study of unpasteurized ciders and cider musts obtained from different cider houses from northwestern regions of France reported 15 yeast species among 208 picked isolates [29]. The main species in this study was *Saccharomyces bayanus* accounting for 34.5% of the isolates, followed by *Saccharomyces cerevisiae*, *Lachancea cidri*, *Dekkera anomala* and *Hanseniaspora valbyensis* representing 16%, 15%, 10.5% and 6.5% of the isolates, respectively. The proportions of each of the 10 other species, i.e., *Candida oleophila*, *C. sake*, *C. stellate*, *C. tropicalis*, *H. uvarum*, *Kluyveromyces marxianus*, *Metschnikowia pulcherrima*, *Pichia delftensis*, *P. misumaiensis* and *P. nakasei*, never exceeded 3.5% of the total isolates. Yeast diversity was higher in cider musts than bottled ciders. Regarding the dominance of *S. bayanus*, the same observation was made in natural cider from Asturias (Spain) [30]. *Saccharomyces bayanus* was the predominant species from the beginning to the middle steps of the fermentation process, accounting for up to 41% of the picked isolates, whereas *S. cerevisiae* took over the process in the final stages of fermentation. *H. valbyensis* was always present at the end of fermentations regardless of the fermentation process used. The variations in the proportions of the different identified yeasts are connected to the occurrence of a sequential succession of yeast species throughout the cider-making process. Morrissey et al. thus identified



three phases in the cider process based on the dominant yeast species present [28]. The first phase, which they called ‘the fruit yeast’ phase, is dominated by *Hanseniaspora uvarum*/*Kloeckera apiculata* yeasts, along with a few *S. cerevisiae* yeasts [14,28]. The second phase, or ‘fermentation phase’ where the alcoholic fermentation occurs, is characterized by the replacement of oxidative or slightly fermentative non-*Saccharomyces* yeasts by the strong fermenting *Saccharomyces* yeasts, such as *S. bayanus* and *S. cerevisiae*. The last ‘maturation phase’ is dominated by *Brettanomyces*/*Dekkera* yeasts. The yeast population fluctuates from one year of production to the next [31]. This is visible in the variations in the proportions of the main yeast species constituting a resident mycoflora throughout cider cellars and by the intermittent apparition of some species constituting a ‘transitory mycoflora’.

## 2.2. Bacterial Diversity

Bacteria are present from the apple flowers to the final product (Table 1). In 2013, Shade et al. studied the apple flower microbiome by pyrosequencing and described the presence of diversified bacterial communities evolving differently from the bud to the fruit [32]. This study highlighted that apple flowers carry bacteria that will be involved in the process of cider or vinegar making (mainly *Lactobacillaceae* and *Acetobacteraceae* families, respectively). Surprisingly, bacteria from *Deinococcus-Thermus* phylum were found in abundance. This phylum was not known to be related to fruit crop. *Enterobacteriaceae*, commonly isolated on apple fruits, were present at every stage of the flower maturation.

**Table 1.** Bacterial diversity found in apple juice-related products.

Family	Origin	Genus/Species	References
<i>Lactobacillaceae</i>	Apple flower Fresh-cut apple Cider	<i>Lactobacillus brevis</i>	[24,33–36]
		<i>Lactobacillus (para)collinoides</i>	
		<i>Lactobacillus casei</i>	
		<i>Lactobacillus diolivorans</i>	
		<i>Lactobacillus hilgardii</i>	
		<i>Lactobacillus sicerae</i>	
		<i>Lactobacillus suebicus</i>	
		<i>Pediococcus ethanolidurans</i>	
		<i>Pediococcus parvulus</i>	
<i>Leuconostocaceae</i>	Cider	<i>Oenococcus oeni</i>	[37,38]
		<i>Leuconostoc mesenteroides</i>	
<i>Acetobacteraceae</i>	Apple flower Apple cider vinegar	<i>Acetobacter</i> sp.	[17,32]
		<i>Komagataeibacter</i> sp.	
		<i>Gluconobacter</i> sp.	
<i>Sporolactobacillaceae</i>	Cider	<i>Sporolactobacillus</i> sp.	[39]
<i>Sphingomonadaceae</i>	Cider	<i>Zymomonas mobilis</i>	[40,41]
<i>Enterobacteriaceae</i>	Apple surface	Coliforms	[24,27,32]
	Apple flower	<i>Enterobacteriaceae</i> <sup>a</sup>	

<sup>a</sup> Genus unspecified.

In 2015, Graça et al. detected principally mesophilic and psychrotrophic microorganisms on fresh cut apple while coliforms and LAB were isolated on apple flowers [24]. Focusing on cider apples, Alonso et al. used PCR-DGGE to study the native microbiota of five apple varieties commonly used in the Asturian cider-making process. Predictably, *Enterobacteriaceae* were present due to the ubiquity in nature of this genus, but bacterial species usually associated with spontaneous fermentation were not [27]. The apple surface microbiota may not be a determinant in the fermentation process. The microbiota of apple cider is strongly influenced by other factors such as harvest techniques, quality sorting and storage. In 2004, Keller et al. brought to light the influence of picking techniques on the microbiota [42]. Cider apples picked from the ground after their fall bring more bacterial diversity

than those tree harvested. After grinding, no difference between bacteria counts were found, whether they were stored or not. However, significant differences in bacterial counts between apple varieties were identified.

Bacterial starters do not exist yet in cider; thus, Sanchez et al. investigated LAB prevalence during the malolactic fermentation in Asturian cider cellars in order to find the most efficient fermentative strains [33]. They mostly isolated strains of *Lactobacillus brevis* and *Oenococcus oeni*. This last species is already known to be very tolerant to low pH and to the presence of alcohol [38]. According to a fermentation capacity evaluation of the selected strains, *O. oeni* strains were the most efficient. Salih et al. also highlighted the importance of *O. oeni* during the malolactic fermentation, and the presence of *Lactobacillus brevis* in some of the ciders tested [37]. Different behaviors of the LAB flora depend on the kind of apples used for cider-making (sweet cider apples, sweet dessert apples, bitter cider apples). The influence of the geographical origins of the indigenous cider LAB was determined by Sanchez et al. using the RAPD (Random Amplification of polymorphic DNA) technique on *O. oeni* strains. Five distinct groups, specific to only one producing area, were identified and had an identical RAPD profile. This significant result brought to light the link between *O. oeni* strains and their geographical origin [33]. A recent study focusing on the biogeography of *O. oeni* confirmed the importance of genetic adaptation of this species in cider and also highlighted that *O. oeni* from wine or from cider were genetically different [38]. The first genome of *O. oeni* has been sequenced and annotated in 2005 [43]. Many studies have investigated the genome of this bacterium and have shown that *O. oeni* strains from wine or cider present a different genomic content [44–46]. A recent study of Sternes et al. analyzed the pan-genome of *O. oeni* with 191 strains, of which only four have been isolated from cider [46]. They showed again that three out of four of the cider isolates cluster closely together. The presence of neighboring wine-derived strains suggests that information from additional strains isolated from cider is required before any conclusion regarding the possibility of a cider-specific subset of *O. oeni* can be reached. The other source of genomic data from LAB isolated from cider is related to their technological or probiotic potential [47,48].

*Lactobacillus* sp. and *Oenococcus* sp. are the most common LAB identified in apple juice byproducts. In apple cider vinegar, which is the result of acetic fermentation, both of them were detected, even if acetic acid bacteria, such as *Acetobacter* sp., *Komagataeibacter* sp. or *Gluconobacter* sp., were the most abundant [17]. In 2010, Sanchez et al. studied the LAB diversity during malolactic fermentation in an industrial cider [13]. Using molecular tools, such as 16S rRNA gene sequencing, *Lactobacillus collinoides*, *O. oeni*, *Pediococcus parvulus* and, with minor content, bacteria like *L. casei* or *P. ethanolidurans* were identified. Acetic acid bacteria are necessary for vinegar production, but can ruin cider production. In contrast, LAB are essential in malolactic conversion during cider production, but some can damage the product by producing spoilage compounds.

### 2.3. Factors Influencing Microbial Diversity

Variations in the microbial ecosystem of ciders are associated with several factors, from the orchards to the final product. First, microbial diversity is determined by the growing conditions of the fruits such as the apple varieties, the climate and the production process. The cultivation practices have an impact on the fruit microbial composition in terms of abundance and diversity. Organic and conventional apple bacterial communities were shown to be significantly different [49,50]. The organic apple phyllosphere displayed higher numbers of bacteria than the conventional apple phyllosphere. A comparison of integrated and organic growing systems for Golden Delicious apple production also revealed significantly higher frequencies of filamentous fungi, greater abundance of total fungi and of taxon diversity in organic apples than in integrated apples [51]. The crop management methods thus influence the microbial communities associated with the surface of apple fruits used for cider production. The apple variety also has an influence on the microbial composition of the fruits. Keller et al. showed that significant differences exist in total aerobic bacterial and fungal populations among apple varieties in relation to their pH, Brix and titratable acidity [42]. The apple

varieties with the lowest titratable acidity, highest pH and highest Brix have the highest microbial concentrations ( $\geq 2.5$  log CFU/g). The method of harvesting also plays a role in microbial diversity. Microbial populations on apples, in pomace and in cider are higher when apples are harvested off the ground rather than tree-picked. In the final cider, the average aerobic plate counts for all pooled varieties tested in the ground-harvested group was 4.89 log CFU/g compared with 2.88 log CFU/g for the fresh tree-picked group [42].

After fruit harvesting, the cider process modulates the microbial composition of ciders. The culling of apples result in ciders with higher microbial numbers than those made from uncultured apples [42]. A strong link exists between the temperature profile of the cider fermentations and the yeast population dynamics of the predominant yeast species, present within the fermentations [28]. Another piece of research also showed that the musts obtained by pneumatic pressing were dominated by non-*Saccharomyces* yeasts (*Hanseniaspora* genus and *Metschnikowia pulcherrima*), whereas in the apple juices obtained by traditional pressing, *Saccharomyces* together with non-*Saccharomyces* were always present [30].

Cider processing facilities and cellars walls, floors and surfaces also constitute reservoirs of bacteria and fungi throughout the cider process. For example, one source of *S. cerevisiae* yeasts appears to be the process utensils, the press house and the vat-house, in which this resident flora can be found even six months after the last pressing [28].

Even if microbial reservoirs are broad, the microbial diversity and microflora successions also greatly depend on the aptitudes of the bacterial and fungal strains to resist or adapt to the process conditions such as depletion in oxygen levels, sulfites presence, CO<sub>2</sub> and alcohol productions and essential nutrients' availability. It also depends on the differences in their specific growth rates, in their sugar uptake capabilities, on inter-specific competition, cell death, flocculation and/or natural sedimentation characteristics [52].

### 3. Microbial Contribution to Cider Organoleptic Quality

#### 3.1. Yeast Contribution

During alcoholic fermentation, many byproducts such as esters, higher alcohols and phenolic compounds are produced as secondary metabolites. Esters provide mainly fruity and floral notes; higher alcohols provide 'background flavors'; whereas the phenolic compounds can generate interesting or unpleasant aromatic notes. Esters are the main volatile compounds in cider behind ethanol [53]. They are characterized by a high presence of ethyl acetate, which alone can represent up to 90% of the total esters [54,55]. The amount of acetates produced by yeasts seems to be strongly related to the nature of the strains leading to alcoholic fermentation: *Saccharomyces* sp. produce fewer acetate amounts than non-*Saccharomyces* yeasts. Comparing the potential of *H. valbyensis* and *S. cerevisiae* to produce volatile compounds, Xu et al. [55] showed that *H. valbyensis* yielded higher concentrations of ethyl acetate and 2-phenylethyl acetate, while *S. cerevisiae* kept more free (non-esterified) isoamyl alcohol and isobutanol. A small variation in the ester concentration of ciders may have significant consequences on their final sensory quality [56]. Most of the esters are responsible for the fruity characteristics of ciders. However, an excessive amount of ethyl acetate may lead to an unpleasant smell of solvent.

Higher alcohols are directly derived from the metabolism of yeasts. They are synthesized during fermentation from oxo-acids originating in amino acids and sugar metabolism [57]. In ciders, they are mostly represented by isopentanol (2- and 3-methylbutanol) followed by isobutanol, propanol, butanol or hexanol [58]. Although they constitute a relatively low amount of the total substances, higher alcohols may greatly influence sensory characteristics. Rapp and Mandery [59] found the total higher alcohols in wine to be in the range  $80 \pm 540$  mg/mL; concentrations up to 300 mg/L contribute to pleasant flavor, but concentrations above 400 mg/mL provoke unpleasant flavor and harsh taste. Some higher alcohols, particularly iso-amyl alcohol, contribute to unpleasant flavor [60], although a positive correlation has been reported between n-butanol and the aroma quality of apple juice [61].

The third class of secondary products, i.e., the phenolic compounds, also have important effects on the sensory properties of apple ciders by either their content or their profile. These compounds derived from raw material have an impact mainly on color, bitterness, and astringency [62]. High molecular weight procyanidins in ciders are known to contribute to astringency, whereas the smaller compounds contribute to bitter taste [63–65]. Simultaneously, they influence the sweetness and sourness, thus further highlighting their importance in overall flavor development [64]. In addition to the non-volatile phenolic compounds, the volatile phenolics mainly formed by enzymatic decarboxylation during fermentation contribute to aroma [66].

It has been reported that during the early stages of fermentation, excess growth of the apiculated yeast *Kloeckera* can generate high levels of esters and volatile acids [67]. In wine, the aromatic profile is negatively influenced by the yeast *Brettanomyces/Dekkera* and is characterized by mousy, medicinal, wet wool, burnt plastic or horse sweat smells [68]. Buron et al. have shown that *Brettanomyces/Dekkera* cider strains were able to produce 4-ethylcatechol, 4-ethylphenol and 4-ethylguaiacol from caffeic, *p*-coumaric and ferulic acids, respectively [69]. These volatile phenols are associated with organoleptic defects. In contrast, in some beers, this yeast is considered essential and beneficial [70]. In wine- and cider-making on an industrial scale, the control of *Brettanomyces/Dekkera* is usually achieved through the addition of sulfur dioxide (SO<sub>2</sub>) to the fermentation medium [71]. In cider-making, the concentration of SO<sub>2</sub> is in the range of 50–150 mg/mL at pH 3.0–3.8, not exceeding 200 mg/mL in total [72]. However, some strains of *Brettanomyces/Dekkera* are naturally resistant to SO<sub>2</sub>, and elimination of this yeast by physical treatments (filtration) has a limited efficiency (due to the cell size of this yeast) and does not prevent subsequent recontamination.

### 3.2. Bacterial Contribution

Transformation of malic acid, lowering total acidity, is the major organoleptic change induced by LAB. During MLF, the strong green taste of malic acid is replaced by the less aggressive taste of lactic acid [73]. However, LAB are also responsible for other changes in aromas increasing flavor complexity, involving changes of fruity, flowery and nutty flavors, as well as the reduction of vegetative/herbaceous aromas by reduction of acetaldehyde metabolism [74–76].

*Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* are genera of special interest as they are able to survive cider environments (low pH, high ethanol content and low nutrients). Research focuses on the contribution of *O. oeni*, but other genera, particularly *Lactobacillus* species, should not be underestimated [77]. In wine, it is well known that some varietal aromas revealed during alcoholic fermentation by yeast disappear or change after malolactic fermentation [73]. For example, the concentration of some esters can be either increased or decreased by MLF, according to the type of bacterial strain used [78]. Apart from esters, aroma compounds such as higher alcohols, fatty acids, lactones and sulfur and nitrogen compounds can be produced by LAB [77].

LAB contribution to aromatic profiles of ciders has been explored less than it has been in wine. A few studies are available, principally linked on the use of *O. oeni* strains as starters rather than studying LAB metabolism in the cider environment. In wine, LAB contribution is focused on citric acid metabolism that induces the production of compounds linked to buttery descriptors: diacetyl, 2,3-butanediol and acetoin [79]. Together with acetic compounds, citric acid degradation involves the production of acetic acid that can significantly modify the aromatic profile. Citric acid metabolism with the production of diacetyl cannot be responsible for the whole panel of flavor modifications, and the mechanisms should be further studied.

Along with the favorable sensory changes that can occur during cider elaboration, LAB can be also responsible for undesirable reactions. The frequent cider alteration known as ‘piqûre acroléique’ is mainly caused by a heterofermentative LAB commonly encountered in cider, *Lactobacillus collinoides* [80,81]. In apple-derived products, this alteration results from glycerol degradation to 3-hydroxypropionaldehyde (3-HPA) under the action of *L. collinoides* via the diol-dehydratase enzyme. In addition to *L. collinoides*, some other cider species, like *L. hilgardii* [34] or *L. diolivorans* [82], are able to produce 3-HPA. Glycerol is



one of the major products of yeasts metabolism during cider alcoholic fermentation and is important for the sensorial quality of fermented beverages. Due to its high instability, during the distillation process, the 3-HPA is transformed by dehydration [83] in acrolein, a lachrymatory chemical generating a peppery flavor, which can spoil the product, giving a bitter taste [84,85].

One major spoilage microorganism is the Gram-negative, facultative anaerobic bacterium *Zymomonas mobilis* isolated from various alcoholic beverages, including ciders, beers and perries. *Z. mobilis* is a remarkable bacterium and a very promising microorganism for industrial ethanol production because its catabolism follows the Entner–Doudoroff pathway, thus giving a near-theoretical yield of ethanol from glucose, fructose and sucrose, the only carbon and energy sources that support its growth [40]. As a cider spoilage microorganism, growth of *Z. mobilis* is correlated with the production of large quantities of acetaldehyde along with an almost explosive production of gas and a marked turbidity of the product, an alteration known as ‘framboisé’ in French ciders or ‘cider-sickness’ in English ciders [41,86]. Associated with these symptoms is a marked change in the flavor of the beverage, the original fruity character being lost or hidden by a strong and characteristic taste, reminiscent of raspberry. Malolactic fermentation (MLF) is considered to enhance the risk of ‘framboisé’, and Bauduin et al. [41] have shown that the relationship between MLF and ‘framboisé’ is mainly associated with the increase of pH correlated with the conversion of malic acid to lactic acid rather than with nutritional factors produced by LAB. In fact, the amount of residual nitrogen in cider appears to be the main factor controlling the growth of *Z. mobilis*, and thus, a solution for the prevention of this alteration consists of reducing the amount of residual nitrogen as soon as possible [41].

Therefore, a greater knowledge of cider LAB flora and their metabolisms in a cider environment could provide laboratory and practical cellar tools for a better control of cider quality.

#### 4. Safety Assessment of Fermented Apple Beverages

Fermented foods and beverages are known to be safer than unfermented counterparts. The improved food safety arising from fermentation is largely due to LAB, a predominant group of organisms in most fermented foods and beverages. Occasionally, bacterial pathogens such as *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus*, originating from orchard soil, farm and processing equipment or human sources, may occur in apple juice. However, both apple juice and fermented cider contain organic acids, mainly malic acid ( $\cong 5$  g/L) in apple juice and lactic acid (3–4 g/L) in fermented cider, generating acidity (pH level ranging from 3.0–3.5 and 3.3–4.0, respectively) that usually prevents the growth of these pathogens, which can survive for only a few hours. The growth and metabolism of LAB usually inhibit the growth of normal spoilage flora of the matrix and of any bacterial pathogens that it may contain. Therefore, apple cider is traditionally not regarded as a potentially hazardous food [87]. However, the monitoring of food-borne hazards in cider such as the pathogenic bacteria *E. coli*, protozoan *Cryptosporidium*, biogenic amines or mycotoxins still requires vigilance on the part of cider producers.

##### 4.1. Biogenic Amines

Biogenic amines (BA) are low molecular weight organic bases with an aliphatic, aromatic or heterocyclic structure frequently occurring in foods and beverages involving fermentation or the ripening process. The formation of these molecules is achieved through the removal of the alpha carboxyl group from amino acids [88]. The most abundant BA found in foods are histamine, tyramine, putrescine, cadaverine and phenyl ethylamine. In fermented beverages, such as beer, wine and cider, production is influenced by microorganisms present [88,89], environmental factors such as pH, ethanol [90,91], sulfur anhydride level [92], raw material quality and fermentation, as well as technological conditions [90,93]. Consumption of food containing high level of BAs can induce adverse reactions such as headache, hyper- or hypo-tension and rashes. Such disorders may become serious especially for consumers whose detoxification system is impaired either by genetic disorders or medical

treatments [89]. Histamine and tyramine are considered as most toxic and particularly relevant for food safety, while putrescine and cadaverine are known to potentiate these effects [94].

In cider, as microbiological stabilization is not performed after MLF, indigenous heterofermentative LAB constitute the predominant flora capable of promoting the production of BAs [36,39,95]. As shown in Table 2, among LAB, *Oenococcus* and *Lactobacillus* were found to be the most representative genera of BA producers in cider.

**Table 2.** Potential bacterial species producers of biogenic amines in Spanish and French ciders.

Biogenic Amine	Producer	References
Histamine	<i>Lactobacillus paracollinoides</i>	[35,86,94]
	<i>Lactobacillus hilgardii</i>	
	<i>Lactobacillus diolivorans</i>	
	<i>Lactobacillus collinoides</i>	
	<i>Oenococcus oeni</i>	
Putrescine	<i>Lactobacillus collinoides</i>	[39,89]
	<i>Oenococcus oeni</i>	
	<i>Lactobacillus brevis</i>	
	<i>Lactobacillus mali</i>	
	<i>Leuconostoc mesenteroides</i>	
	<i>Pediococcus parvulus</i>	
Tyramine	<i>Lactobacillus paracollinoides</i>	[35,86,93]
	<i>Sporolactobacillus</i> sp.	
	<i>Lactobacillus brevis</i>	
	<i>Lactobacillus diolivorans</i>	
	<i>Oenococcus oeni</i>	
	<i>Pediococcus parvulus</i>	

Studies conducted on commercial cider from Spain and France revealed the presence of BA in almost 90% of the analyzed products with a higher prevalence of tyramine, histamine, putrescine and cadaverine among other amines [89,95]. Nevertheless, BA content of cider seems to be lower than that detected in other fermented foods and beverages [88]. Some differences in amount and composition were also found between French and Spanish samples. More precisely, cadaverine and putrescine were detected at a maximal concentration of 34 mg/L in 20% and 57% of Spanish cider samples, respectively, while only in trace amounts in only a third of French cider samples (1 mg/L). Tyramine was the most frequently detected BA in French samples (present in 70% of samples in concentrations below 14 mg/L). Histamine was detected at relatively low levels in both French and Spanish samples (26% of total samples, below 16 mg/L) [89,95,96]. As mentioned by Ladero et al., the characteristics of the apple variety used and/or the different elaboration processes, as well as possible microbiota differences could explain the differences [89]. The global amount and profile of BA produced do not appear to be driven by cider-making steps and types of press [95]. Therefore, some strategies have been proposed to decrease the formation of BA, such as (a) reducing amino acid precursor levels (generally decreasing with fruit ripening), (b) limiting the growth of spoilage bacteria, (c) inoculating starter cultures without amino acid decarboxylase and (d) inoculating biogenic amine-degrading microorganisms [94,97].

#### 4.2. Mycotoxins

Mycotoxins are secondary metabolites of filamentous fungi mainly triggered by *Aspergillus*, *Fusarium* and *Penicillium* genera [98]. In apple and apple-derived products, patulin represents the most relevant mycotoxin. The toxin is an unsaturated heterocyclic lactone toxin produced by a wide range of mold species [99]. Among these species, *P. expansum*, as the main pre-harvest and post-harvest contaminant in pomaceous fruits (apples and pears), is considered as the major source of patulin in these fruits [100]. The level in food and beverages is regulated in Europe by the European Commission

and in the United States by the U.S. Food and Drug Administration (FDA) to a maximum acceptable concentration of 50 µg/L for fruit juices and derived products (including cider) [101]. Indeed, Michigan apple cider mills were analyzed for patulin concentration. The mycotoxin was detected in almost 20% of cider mill samples with 2% of samples having concentration higher than 50 µg/L [102]. Temperature, activity of water ( $a_w$ ) and pH were found to influence *P. expansum* growth, as well as patulin production. The fungi is able to produce the toxin around 16 °C. *P. expansum* can produce the mycotoxin only at an  $a_w$  of 0.99, which is the approximate  $a_w$  of fresh fruits. Finally, patulin production was found to be optimum at pH 4 [103]. Apple contains natural acids (citric and malic acids) that lead to the reaching of these optimal conditions (pH of fruit varies from <2.5–5) [104]. It is commonly admitted that the toxin is generally unstable during fermentation, so that products such as cider are usually free of patulin. In a recent study, the level of patulin in contaminated musts was shown to have decreased six-fold after two days of fermentation [105]. Reports of patulin in cider are likely due to the adjunction of apple juice to produce ‘sweet cider’ or low-fermented cider [102].

#### 4.3. Pathogens

As previously mentioned, unpasteurized apple cider is historically considered to be a safe product, free of microbial pathogens due to its acidic level and to the fermentation process. However, some bacterial and parasitic pathogens can survive and may remain infectious [106]. To date, enterohemorrhagic *E. coli* (EHEC) serotype O157:H7, as well as the protozoan *Cryptosporidium parvum* have been linked to several outbreaks since the 1980s due to apple cider consumption [106]. Nevertheless, it is important to notice that such outbreaks only occurred in North America and mainly in unfermented apple ciders [106]. European apple cider has never been implicated in any outbreaks of this kind due to alcoholic fermentation process byproduct, ethanol, which is toxic for most potential pathogens in cider [107]. EHEC O157:H7 is known to have a fecal origin and may contaminate apples, juice and cider directly from animal/human feces or by indirect contact (equipment, contaminated water, etc.) [108]. *Cryptosporidium* spp. is an intracellular parasite with an infectious stage known as oocysts. Oral transmission of the parasite is facilitated by the ability of oocysts to survive for weeks to months in the environment. The study of contamination sources in unpasteurized apple cider revealed that the parasite is found in washed apples, water, fresh and finished cider [108]. Kniel et al. studied the potential of malic acid, as well as hydrogen peroxide to reduce the infectivity of *C. parvum* in apple cider. Interestingly, infectivity was completely inhibited by incubation of oocysts in apple cider plus 0.025% H<sub>2</sub>O<sub>2</sub> and inhibited (up to 88%) by the addition of 5% malic acid [109].

### 5. Functional Improvement of Apple Fermented Beverages

#### 5.1. Control of the Microbial Ecosystem to Improve or Modulate Cider Quality

##### 5.1.1. Rational Design of Starter Cultures

The selection of appropriate starter strains is key in the control of the cider fermentation process and characteristics of the final beverage. Microbial starters, especially *O. oeni*, are less used in cider production than in wine production, but their role might be crucial for the quality of the final product [110]. This leads to many studies focusing on the selection of microbial starters [33,111], their improvement [112,113] and the use of other LAB than *O. oeni* [114]. The use of isolated strains of *S. cerevisiae* is an interesting strategy for maintaining the quality and reproducibility of fermented beverages. This is especially true for the Champenoise method, typical of Asturian PDO ciders, and based on a secondary fermentation in bottle. The screening and the selection of local yeast strains is believed to be more effective than using commercial starters, as these endemic strains are potentially better acclimated to the environmental conditions than industrial starters [115]. These authors thus proposed a methodology for the rapid screening and selection of autochthonous yeast strains based on their oenological and technological properties. The ciders obtained with the

selected yeast strains were scored as good after sensory analysis. The choice in species driving the fermentation is important for technological purposes and also for the aroma profile development in cider. For example, the presence of *Hanseniaspora* sp. yeast strains during apple fermentation results in the production of considerable amounts of esters and alcohols, contributing to fruity sensory notes, compared with apple musts fermented only with *Saccharomyces* sp. yeasts, which provide rather neutral sensory notes [116]. The fermentation performance has also been improved by the use of a hybrid strain between *S. eubayanus* and *S. cerevisiae* [111]. Recently, in order to control the proliferation of *Brettanomyces*/*Dekkera* in wine, Ngwekazi et al. [71] have identified and characterized killer toxins secreted by non-*Saccharomyces* yeasts related to wine. Their results, although preliminary, show that killer toxins have a high potential to control the population of large numbers of *Brettanomyces*/*Dekkera* strains. These results are especially encouraging, as none of the killer toxins characterized inhibit the fermentative yeast *Saccharomyces*.

Another characteristic to be considered for the bacterial strains used in the cider fermentation process is their aptitude in resisting bacteriophages (phages). The presence of lytic or lysogenic phages of *Oenococcus* has previously been described in wine [117]. Recently, Constanti et al. characterized *O. oeni* bacteriophages and the related implications for malolactic fermentation in wine [118]. They reported that pH and ethanol affect the lytic activity of *Oenococcus* phages, especially when wine alcohol content is low. The presence of phages in cider has not yet been investigated. It is thus a point of interest that should be examined, as anti-*Oenococcus* phages could be at the origin of cider fermentation problems by disturbing the malolactic fermentation driven by phage-sensitive *Oenococcus* strains. Still, phages could be used as antimicrobial agents against spoilage and pathogenic bacteria in ciders and therefore help in controlling the safety and quality of these fermented beverages. Phage therapy in the food industry has been extensively studied [119]. However, phage applications to apple fermented beverages are scarce. One main obstacle to their efficient antimicrobial action would be their potential sensitivity to acidity found in apple products [120].

#### 5.1.2. Control of the Fermentation Process Parameters

Spoilage and pathogenic flora in apple juice and by extension in apple fermented beverage can be reduced by physical methods, which have already been reviewed [121] and will not be further discussed. The control of the fermentation process parameters such as the time of inoculation of mixed cultures, the temperature of fermentation steps in the process or prefermentative steps is a key element in the modulation of the cider ecosystems throughout processing and thus of the quality of the final product. Aroma production during cider fermentation is greatly dependent on the yeast species in the presence and their sequential succession throughout the process. A study of the co-culture of *Wickerhamomyces anomalus* and *S. cerevisiae* showed that the association could help improve the quality and add complexity to the cider [122]. Controlling the strain association parameters during the fermentation process, i.e., the inoculation time and the sequential or simultaneous mixed cultures, is crucial for the optimization of the desired kind of cider. In the same way, the fermentation of a vegetable juice using a mixed culture of *S. cerevisiae* and *L. plantarum* resulting in an enhancement of the nutritional content of the final beverage [123] emphasizes the feasibility of the chosen co-fermentation by the selection of the right microbial associations for designing new functional apple fermented beverages.

Yeast metabolism is greatly dependent on the temperatures applied during the fermentation process. Peng et al. [124] have shown that variations of the fermentation temperature have a direct influence on the aromatic profile of the final cider. In their study, the ciders fermented at 20 °C seemed to result in the best acceptance by the consumer and displayed the highest aromatic characteristics. This is probably due to modifications in the microbial metabolism that result in variations in the production of esters, volatile compounds and alcohols according to the fermentation temperatures. Variations in the fermentation temperature are nonetheless to be considered with care, as the increase of temperature can also lead to the formation of undesired compounds by the expression of unsuitable



microbial metabolic pathways [124,125]. In the same way, fruit processing, pectinolytic enzyme application, cell yeast immobilization on alginate and the type of fermentation have all a significant influence on the antioxidant capacity, polyphenol profile and volatile composition of ciders [126]. Prefermentative treatments such as pulp fermentation induced the formation of higher amounts of ethanol, procyanidins B2 and C1, epicatechin and catechin and resulted in a higher antioxidant activity than in non-pulp fermented ciders. Cell immobilization positively affected the ethanol content, but decreased the antioxidant activity of ciders. Ciders obtained with spontaneous fermentation contained more esters and methanol compared to inoculated ciders [126]. The MLF is also a bottle-neck in the cider production process, and one area of research is looking for strategies to control/improve this natural phenomenon [127]. Such process parameters can thus be used as levers to modulate the quality of the final product.

### 5.1.3. Control of Cider Quality by LAB

Microbial quality is obviously microorganism dependent and is highly affected by chemical, physical and biological factors pertaining to the environment. Maintaining microbiological quality and the maximum sensory and nutritional quality of fermented beverages requires a combination of antimicrobial hurdles in order to limit the growth of undesired microorganisms. By producing organic acids as a fermentation metabolite, antimicrobial peptides and hydrogen peroxide, LAB strains may contribute to improving the quality of apple ciders. Bacteriocins are generated from bacteria and, usually, are inhibitory towards phylogenetically-related species. There are only a few reports about the inhibitory activity of bacteriocins against yeasts [128–130]. To our knowledge, the effectiveness of bacteriocins from LAB for controlling the growth of undesirable yeasts in cider or wine has never been studied, although bacteriocins produced by LAB have received considerable attention over the years for their possible use as biopreservatives in food, to reduce the use of chemical preservatives. It could therefore be interesting to screen new bacteriocins from LAB isolated from fermented beverages.

Bacteriocins could also be effective against spoilage bacteria. *L. collinoides* exhibits natural resistance to conditions encountered during the fermentative process [131]. In order to avoid this alteration, the bacteriocin enterocin AS-48, a broad-spectrum antimicrobial peptide produced by *Enterococcus faecalis* [132], was tested against two 3-HPA-producing *L. collinoides* strains causing apple cider spoilage. The two *L. collinoides* strains tested were rapidly inactivated by low concentrations of enterocin AS-48 in fresh apple juice (2.5 µg/mL) and also in Basque cider (2.5–5 µg/mL) [133]. Another classical disorder, which does not affect flavor, is known as ‘ropiness’. This microbiological problem arises when certain bacteria synthesize exopolysaccharides (EPS), thus increasing the viscosity of the cider [134]. The EPS show a large variation in composition, molecular mass and structure and, once secreted into the medium, play an important role in the rheology and texture of fermented beverages, enhancing naturally the texture and viscosity [135]. As a consequence of the increase of viscosity, the cider flows like oil; hence the term ‘ropiness’. In addition to being a biothickener, prebiotic effects of several EPS have been demonstrated [136]; however, despite these interesting properties, a high level of EPS production in cider is unwanted, as it is prejudicial to the organoleptic quality of the product. Although ropiness is mainly caused by some strains of LAB [137–139], it has been shown that one strain belonging to the *Bacillus* genus can be responsible for this alteration [140]. Among the alternative methods suggested to avoid this alteration in beverages, Grande et al. [141] have tested the efficacy of the *E. faecalis* enterocin AS-48 against a slime-producing *B. licheniformis* strain in apple cider. Their results show that enterocin AS-48 is also active against the EPS-producing strain either in culture medium or in apple cider, suggesting a possible use of this enterocin to prevent ropiness. These results are of great interest for the development of tools allowing for the control of undesired bacteria in fermented apple cider.

Thanks to amino oxidase enzymatic activity, some species of LAB appear to be of great interest for the potential control of BA-related health risk. Hitherto, many studies have been conducted with the purpose to identify LAB isolated from fermented foods with BA degrading capability, but only

a few concern fermented beverages. To our knowledge, no study has been conducted on LAB isolated from cider. A collection of 85 LAB isolated from wines, must and lees was screened for their ability to degrade histamine, tyramine and/or putrescine. Twenty-five percent of the LAB were able to degrade histamine, 18% tyramine and 18% putrescine. The strains with highest activity belonged to *Lactobacillus* and *Pediococcus* groups, and most of them were able to degrade simultaneously at least two BAs [108]. In the future, it might be of interest to screen the potential of cider-associated LAB to reduce potential BA level in this beverage.

Large numbers of studies attribute antifungal activity to LAB strains thanks to the production of various organic acids (such as lactic, acetic, caproic, formic, propionic, phenyl lactic and butyric), fatty acids and peptides [142,143]. LAB may represent interesting biological control agents in apple fermented beverages by means other than bacteriocin. The detoxification of patulin through binding to bacterial surface proteins is an example [144,145]. Recently, Zoghi et al. identified two probiotic strains of *L. acidophilus* and *L. plantarum* able to catch the toxin through their surface layer proteins (fructooligosaccharide content). In the best conditions and after six weeks of refrigerated storage, more than 90% of initial patulin were removed from apple juice with no significant difference in organoleptic properties [144].

## 5.2. Health Benefits of Apple Fermented Beverages

Fermented beverages and especially non-dairy probiotic beverages are believed to be the next functional foods for probiotic delivery. Likely candidates are chilled fruit juices or fermented vegetable juices [146]. For the consumer, they present the advantages of lacking dairy allergens such as lactose, containing low cholesterol and having a vegan-friendly status [147]. The health benefits of fermented beverages have been described. The improvement of gastrointestinal health associated with the microbial content of fermented beverage is thought to be responsible for perceived health outcomes. Evidence of the direct or indirect action of the beverage microbiota on gastrointestinal health have been given over the years, even if the mechanisms involved are still unclear for the most part [148]. The health benefits of apple beverages have been the subject of much scrutiny, for many years. For example, apple beverages, including cider, have been shown to have anti-viral properties [149]. Some apple juices are already used as vectors of probiotic lactobacilli strains [150,151]. Several traditional cereal and vegetal fermented beverages are the source of probiotic bacteria [152]. Apple fermented beverages can therefore be sources and vectors of probiotics. Spent cider yeast, a by-product of the fermentation process, was used as a dietary supplementation in a piglet model. This supplementation proved to enhance gut functions and to reduce *Salmonella* and *Escherichia* carriage in porcine gut [153]. Some probiotic potential has also been demonstrated for lactobacilli [48] or pediococci [47,154]. A probiotic beverage from apple fermented with *L. casei* has recently been developed for human consumption [151].

A recent review detailed the role of LAB as an efficient cell factory for the production of functional biomolecules and food ingredients to enhance the quality of cereal-based beverages [155]. These LAB assets could be transposed to apple fermented beverages. They encompass the LAB-mediated inhibition of spoilage or pathogenic microorganisms through antibacterial compound production, the reduction of potential antinutritive factors, the amelioration of the apple fermented beverage nutritional value, the LAB aroma and flavor compound production, the production of EPS related to texture development, organoleptic changes and the prebiotic nature of those beverages, the production of nutraceutical compounds and anti-allergenic biomolecules. Some LAB, isolated from wine or cider, also showed potential intrinsic (without grape/apple matrix) health benefits [156]. For example, *O. oeni* can harbor anti-inflammatory potential [157] or produce EPS [158]. This EPS production could even help with the industrial production of food products containing lyophilized *O. oeni* strains [158]. The EPS production by LAB could also be related to industrial perspectives such as viscosity and mouth feel enhancement properties [159].

The development of functional apple fermented beverages is promising. For example, strategies combining apple juice and a novel whey-based beverage fermented by kefir grains have already been designed [160]. The combination of apple juice and kefir grains resulted in a beverage with high total phenolic content and antioxidant activity.

## 6. Conclusions

This review emphasized the microbial ecosystem of musts and showed how mastering the quality and the safety of cider production is reliant on a better understanding of the mechanisms of LAB and yeast metabolism involved in the transformation of precursors into potent flavor components. The present review further paved the way for the optimization of the industrial scale-up for artisanal cider production using the integrated metabolomics and molecular phylogeny approaches to identify and select strains of LAB, particularly *O. oeni*, to improve the flavor/aroma profiles of ciders. Indeed, although considerable efforts have been made in recent decades to optimize and improve the production of cider, cider remains a product with a great variability related in particular to the notion of ‘terroir’ that can be defined as a homogeneous territory from a soil and climate point of view. Therefore, pedoclimate factors together with indigenous microorganisms may significantly influence the quality and typicity of the cider produced in a specific location. The apple benefits from a good and healthy image that could be combined with new microbial characteristics with a special focus on LAB. Specific research on microbiomes using ‘omics’ tools will give rapid insights into the potential of strains associated with these products. For these reasons, studies on apple fermentation beverages comprise a promising field of research with great potential for available new, healthy and pleasant products on the market.

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## Botanical Innovations

### Apple Cider Vinegar Powder

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## Challenge

Vinegar is one of the oldest fermented foods in the world, with a recorded history dating back 5,000 years. Vinegar is commonly made from a variety of ingredients including apples.

Botanical Innovations has been producing an apple cider vinegar using a traditional slow fermentation process for several years. During the fermentation process an active ingredient known as “mother” is naturally formed. This active ingredient in the vinegar is a natural prebiotic that supports the growth of beneficial intestinal microorganisms in the gut. As the number of studies showing the importance of gut bacteria to overall wellbeing continues to grow, so too does the demand for prebiotic products that support the micro flora ecosystem.

Consumers and manufacturers alike, are demanding foods and ingredients that are natural and recognisable. In response, the food industry continues to look for natural solutions to prevent microbes, slow spoilage, preserve colour, keep flavour and maintain texture whilst replacing artificial additives and preservatives.

The technical challenge, which was the focus of several years of Botanical Innovations research and development, was to create a unique Apple Cider Vinegar powder that included the active ingredient “mother”. This was important as it ensured the product kept two distinct properties- as a natural prebiotic and a natural preservative.

## Solution

Botanical Innovations Apple Cider Vinegar powder is slowly and naturally fermented from only Australian grown apples. The raw apple cider vinegar with “mother” is dried using a proprietary technology creating this unique functional ingredient.

The product can be used to add prebiotics and flavour to chips, snacks, and beverages. It is also used in a variety of other food and beverages as a natural preservative and shelf life extender, including baked goods such as bread, biscuits.

## Outcome

Botanical Innovations Apple Cider Vinegar powder proudly won the Food and Beverage Industry Innovative Ingredient Award in 2018.

Botanical Innovations Apple Cider Vinegar powder is now exported to many countries and is also utilised by several domestic customers looking for a clean label prebiotic capable of supporting gut health.

Customers use the product as an ingredient in health foods, dairy products, beverages, bakery goods, nutrition bars, infant foods, and green products. Apple Cider Vinegar powder is also used as a health supplement. New product applications are regularly being discovered, ensuring a growing market opportunity for Botanical Innovations.



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