PROTOCOL AND STATISTICAL ANALYSIS PLAN
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Nitric oxide for preventing and reducing the severity of winter infections in care homes (BEET-Winter): a feasibility trial

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ABSTRACT

Epidemic winter infections cause considerable morbidity and mortality in care homes, compounded recently by COVID-19. Nitric oxide (NO) is a generic antimicrobial with anti-viral (including influenza and CoVs), bacterial, protozoal and fungi/yeast activity.

We are performing a cluster-randomised placebo-controlled feasibility trial of nitric oxide, delivered as beetroot juice (70 ml of 400 vs 0 mg nitrate) given for 60 days in care home residents. The main outcomes are feasibility of recruitment of care homes and residents, adherence to beetroot juice, urinary nitrate, and measurement of an ordinal infection outcome: no infection, uncomplicated infection in care home, infection in care home requiring healthcare support, all-cause hospitalisation and all-cause mortality.

The following protocol and statistical analysis plan describe the trial's rationale, methodology and planned tables and figures and their methods of analysis.

INTRODUCTION

Epidemic winter respiratory infections cause considerable morbidity and mortality in care (residential and nursing) homes. Common viral causes include influenza A/B viruses, parainfluenza virus, respiratory syncytial virus (RSV), rhinovirus and coronaviruses (CoVs: 229E, NL63, OC43, HKU1). Bacterial causes include *Chlamydia pneumoniae*, *Haemophilus influenzae*, *Legionella* spp. and *Streptococcus pneumoniae*.[1] Care homes also have winter outbreaks of gastrointestinal tract, e.g. viral gastroenteritis due to norovirus, urinary tract, and skin and soft-tissue infections.[1] Dominating all of these at present is the ongoing SARS-CoV-2 coronavirus pandemic which has had catastrophic consequences [2] with a third of excess deaths occurring in care homes and a reduction in resident life expectancy by 6 months.[3] Despite significant enhancements made to infection control procedures in care homes (hygiene, personal protective equipment) and prophylaxis with vaccination, SARS-CoV-2 infections continue to occur. Co-located older people in care homes are at high-risk for outbreaks of infectious diseases and yet there are no general antimicrobial measures that have demonstrated prophylactic efficacy against such outbreaks. By example, interventions such as probiotic capsules [4] have failed to demonstrate efficacy.

Nitric oxide (NO) is a generic antimicrobial with substantial *in vitro* and some *in vivo* data demonstrating anti-viral, bacterial, protozoal and fungi/yeast activity.[5] The antimicrobial effects of NO and derivative molecules such as peroxynitrite are mediated by effects on DNA and protein conformation.[5] NO also improves organ blood supply and has pro-endothelial and anti-inflammatory and antithrombotic effects mediated through anti-leucocyte and anti-platelet activity,[6] and these may also contribute to its antimicrobial effects. Antiviral and antibacterial activity has been demonstrated against many of the common causes of respiratory, gastrointestinal and soft-tissue infections, including against influenza and CoVs. Phase II and equivalent-stage clinical trials have supported anti-microbial effects of acidified nitrite on cutaneous viral and bacterial infections, dietary nitrate on oral bacteria, and NO gas against some respiratory viral infections.[5] Further, NO has been shown to improve exercise performance and cognition in older people,[7] potential benefits of relevance to care home residents. Although some common infections have vaccines available (e.g. influenza, SARS-CoV-2), many do not (e.g. RSV) and vaccinations may need to be combined with chemoprophylaxis for effective prevention, especially in a population where immunosenescence is the norm.[8,9] So, NO substrates and donors may be particularly relevant due to their potential generic antimicrobial effects, and especially since resistance against NO appears to be rare [5] in contrast to that occurring with many specific antibacterial agents.[1]

Here we describe a cluster-randomised trial to test the feasibility of administering beetroot juice to care home residents and assess safety and early signals of efficacy and safety.

METHODS

Rationale

The proposed trial was designed on the basis of the following premises: (i) Potentially, NO has broad spectrum antimicrobial affects;[5] (ii) Dietary sources of NO have a very low risk of harm and yet may reduce infections and their severity and so potentially save lives; (iii) Care home diets may not be rich in dietary nitrate; (iv) SARS-CoV-2, respiratory epidemic and norovirus infections in care homes increase during the autumn, winter and spring months; (v) The symptoms of many respiratory infections overlap so it is not possible to reliably distinguish clinically between the causative organism (e.g. influenza virus vs. RSV vs SARS-CoV-2) in the absence of multiplex testing, which is not routinely deployed; (vi) Co-infections caused by two or more pathogens of concern may interact in as yet undetermined ways; and (vii) Most COVID-19 trials have focussed on interventions that are unlikely to have effects on other microbial pathogens.

Trial Design and Oversight

BEET-Winter was designed to evaluate the feasibility of administering a high nitrate diet to UK care home residents and assess safety and early signals of efficacy. It was supported by the National Institute for Health Research (NIHR) Nottingham Biomedical Research Centre and PMB's NIHR Senior Investigator award. The trial was a prospective cluster-randomised, placebo-controlled, blinded endpoint study and assessed pre-exposure prophylaxis; further details are provided in the Supplementary Appendix. The trial was coordinated by the Nottingham Stroke Trials Unit at, and sponsored by the University of Nottingham.

Eligibility

UK care homes are eligible for inclusion if they look after older people (whether of residential, nursing or dual registered), are rated at least 'adequate' (based upon inspections by the English Care Quality Commission) and have a minimum of 18 residents (Supplement). Care homes were not eligible if staff lived in the home – an arrangement which became more prevalent during the COVID pandemic. Residents were eligible for inclusion if they were >=65 years of age; were taking a normal or soft diet; and had tasted the concentrated beetroot juice and agreed to take it daily for two months. Residents were excluded if they were in another randomised intervention trial, consent was not available from them or a consultee if they lacked capacity, were using a thickener with food, were fed via a feeding tube, were using daily antiseptic mouthwash [10] and were unwilling to stop this, currently had an infection requiring hospitalisation, had entered end-stage palliative care, were in the care home for short-term respite care, or were taking beetroot juice daily.

Randomisation and masking

As a cluster-randomised trial, care homes are randomised dynamically using minimisation to balance across important baseline care home characteristics: type (residential vs dual registered or nursing alone), prior SARS-CoV-2 infection in wave 1 of the pandemic, and size ($\leq$32 vs >32 residents). Ten percent of randomisations were based on chance. Randomised homes are assigned in a 1:1 ratio to receive nitrate and usual care vs placebo nitrate and usual care. Residents, care home and trials staff are unaware of the assigned treatments.

Interventions

Provision of nitric oxide (NO) was via 70 ml of nitrate-containing (400 mg) beetroot juice (Beet It Beetroot Juice Sport Shot - 70ml, James White Ltd, Ipswich UK), which

is metabolised to NO *in vivo*, via the nitrate-nitrite-NO pathway [11] and was given once daily for 60 days. Placebo was given in the form of 70 ml of nitrate-free (0 mg) beetroot juice (placebo Beet It Beetroot Juice Sport Shot - 70ml, James White Ltd) given once daily for 60 days. Both active and placebo juices have been used in multiple previous clinical trials and are identical in appearance, smell and taste.[12] Further, both juices contain folate, potassium, vitamin C, fibre and antioxidants but no allergens, and can be stored at room temperature with a shelf life >1 year. Juice is palatable to many, but taste can be masked by dilution in other juices, e.g. orange or apple juice, or consumption through a straw.

Procedures

Trial documents including protocol and training materials are hosted on an open website (https://stroke.nottingham.ac.uk/beet-winter/). Research Ethics Committee/Health Research Authority approval was sought through the IRAS system (project 288542), and a favourable opinion received on 19/11/20 (20/WM/0278). Medicines and Healthcare products Regulatory Agency (national competent authority) approval was not necessary since beetroot juice is a food, not a drug. Care Home staff are trained by the research trial team and approach eligible care home residents for electronic informed consent, or relatives if the resident lacked capacity. Capacity is assessed using the '3 question approach', as used in the RIGHT-2 trial.[13]

Following consent/proxy consent, care home staff submit data online (REDCap, Vanderbilt University, Nashville USA) prior to treatment (baseline), at the time of any outcome event or serious adverse event, at 60 days (end-of-treatment) and at 90 days (final follow-up).

Outcome Measures

Feasibility outcomes include: recruitment of care homes; recruitment of residents; adherence to the intervention (75% of residents take >50%); assessment of background dietary nitrate intake; ability to take juice; assessment of salivary and urinary nitrate concentrations (using Quantofix nitrate/nitrite, Camlab, Cambridge UK) ; ability to measure the ordinal outcome measure; assessment of incident infection rate using the ordinal outcome; estimation of the intra-cluster correlation (ICC).

The efficacy outcome is the most serious outcome from the ordered categorical scale comprising 1) all-cause mortality, 2) all-cause hospitalisation, 3) infection with the resident remaining in the care home but needing healthcare support (e.g. from the general practitioner, 111 call, 999 call/paramedic), 4) infection with the resident remaining in the care home and needing no help, and 5) no infection, at 60 days after randomisation; a further analysis assesses this outcome at 90 days. Other outcomes include the components of the efficacy outcome, efficacy outcome in pre-specified subgroups, time to asymptomatic and symptomatic proven SARS-CoV-2 infection, time to first admission to hospital and its cause, time to death and its cause, frailty index,[14] cognition (6-item test) and quality of life (EQ-5D-5L, EQ-VAS). Dietary nitrate is assessed from photographs of lunch meals before and after eating.[15] Safety outcomes comprise adjudicated serious adverse events.

Statistical analysis

A total of 360 residents will be needed from 30 homes with 12 residents per home (range 12-17) will be needed assuming alpha 0.05, power 0.80, intra-cluster correlation (ICC) 0.01 (Supplementary Table 1).[8] Up to six additional care homes, each with between 4-20 participants, can be added in case some homes drop-out, or if fewer than 12 residents are recruited at some homes.

Care home and resident characteristics and feasibility data will be tabulated as number (%), median [interquartile range] or mean (standard deviation). The

comparative analyses will employ a multi-level ordinal logistic regression model with adjustment for the minimisation factors and individual-level covariates (age, sex) and a random effect to adjust for clustering within care homes. The treatment comparison is presented as an adjusted common odds ratio (with 95% confidence intervals) for a shift in the direction of a better outcome on the ordinal scale.[16-19] Prespecified analyses of the efficacy outcome will be performed in subgroups defined by the adjustment factors: care home type, prior SARS-CoV-2 infection in the care home, number of residents in care home, age, sex, vaccination status. Other outcomes will be analysed using appropriate regression models dependent on data type (binary, categorical, continuous, time to event), adjusted similarly and accounting for clustering within care homes. All P values will be two-sided and shown without adjustment for multiple testing. Analyses will be performed using SAS version 9.4.

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Table 1. Baseline characteristics

	All	Nitrate	Placebo
Care home characteristics			
Homes (%)			
Residential			
Mixed			
Nursing			
Region (%)			
East Midlands			
London			
South-east			
Yorkshire			
Last CQC rating (%)			
Excellent			
Good			
Needs			
Client age group (%)			
Older adults, 65+ years			
Mixed, 18+ years			
Younger adults, 18-65 years			
Number of residents			
Number of registered nurses			
Number of non-nurse carers			
Ratio residents to staff			
Staff vaccinated against flu (%)			
Taken part in previous research (%)			
Resident characteristics			
Number			
Age (years)			
>=70 (%)			
Sex, female (%)			
Race-ethnicity, non-white (%)			
Care home (%)			
Residential			
Nursing			
Time in home (days) [IQR]			
Advance directive - no hospitalisation (%)			
Do not attempt resuscitation order (%)			
Medical history (%)			
Blood, e.g. lymphoma, myeloma			
Brain, e.g. PD, MS			
Cancer, under therapy			
COVID-19			
Diabetes mellitus			
Dementia			
Headache/migraine			
Heart, e.g. heart failure			
Heart attack			
Hypertension, on tablets			
Hyperlipidaemia, on tablets			
Kidney disease, chronic			
Kidney stones			
Leg ulceration, current			
Liver, e.g. hepatitis, cirrhosis			
Lung, e.g. asthma, COPD			
Pneumonia			

Stroke			
Urinary catheter, current			
Urinary tract infection			
Interventions (%)			
Steroid tablets, current			
Vitamin D supplementation			
Vaccinated against influenza (%)			
Weight (kg)			
Height (m)			
Body mass index ($kg.m^{-2}$) †			
Number of risk factors (/11) †‡			
Outcome scales			
Clinical frailty scale			
Barthel index (/100)			
6 item cognition (/28)			
Quality-of-life, EQ-VAS (/100)			
Nitrate			
Urinary			
Salivary (mg/L)			
Nitrite, salivary (mg/L)			

† Calculated
‡ Sum of (age>70) + Blood + Brain + Cancer + Diabetes + Heart + Kidney + Liver + Lung + Steroid + (BMI>40)

Table 2. Salivary nitrate and nitrite, and urinary nitrate at baseline and whilst taking beetroot juice
Data are median [interquartile range]; comparison by van Elteren's test including adjustment for baseline. Analyses do not take account of cluster randomisation.

	Baseline		On juice		Difference (95% CI)	P
	Nitrate	Placebo	Nitrate	Placebo		
Nitrate, urine						
Nitrate, saliva (mg/L)						
Nitrite, saliva (mg/L)						

Table 3. Vaccination status against SARS-CoV-2
Data are number (%).

Vaccinated against SARS-CoV-2 (%)	Day 0	Day 60	Day 90
First vaccination (%)			
Pfizer			
AstraZeneca			
Moderna			
Time from first vaccination			
Second vaccination (%)	0		
Pfizer	0		
AstraZeneca	0		
Moderna	0		
Time from second vaccination	0		

Table 4. Clinical outcomes

Data are number (%), median (interquartile range] or mean (standard deviation). Comparisons adjusted for clustering, age, nursing home type.

	Nitrate	**Placebo**	**Unadjusted**		**Adjusted**	
			OR/MD	**p-value**	**OR/MD**	**p-value**
Residents			-	-	-	-
Efficacy outcome						
Worst	-	-	OLR OR	p	OLR OR	p
No symptoms			-	-	-	-
Symptoms of infection			-	-	-	-
Symptoms → healthcare advice			-	-	-	-
Hospitalised, all cause			-	-	-	-
Died, all cause			-	-	-	-
Secondary outcomes						
First event	-	-	OLR OR	p	OLR OR	p
No symptoms of infection			-	-	-	-
Symptoms of infection			-	-	-	-
Symptoms → healthcare advice			-	-	-	-
Hospitalised, all cause			-	-	-	-
Died, all cause			-	-	-	-
First hospitalisation or death, time to			CPHR HR	p	CPHR HR	p
First infection, time to			CPHR HR	p	CPHR HR	p
Respiratory tract			BLR OR	p	BLR OR	p
Influenza †			BLR OR	p	BLR OR	p
COVID-19 †			BLR OR	p	BLR OR	p
Norovirus †			BLR OR	p	BLR OR	p
Urinary tract †			BLR OR	p	BLR OR	p
Cutaneous †			BLR OR	p	BLR OR	p
Other			BLR OR	p	BLR OR	p
Number of infections			PR OR	p	PR OR	p
Disposition (%)			CST	p	-	-
Care home			-		-	-
With relative/friend			-		-	-
At another home			-		-	-
In hospital			-		-	-
Died			-		-	-
Clinical frailty index CFI (/9)			OLR OR	p	OLR OR	p
Barthel index, BI (/100)			MLR MD	p	MLR MD	p
6 item cognitive impairment, 6CIT (/28)			OLR OR	p	OLR OR	p
Quality of life, EQ-VAS (/100)			MLR MD	p	MLR MD	p
Tolerability, >70% of shots			BLR OR	p	BLR OR	p
Vaccinated against SARS-CoV-2			BLR OR	p	BLR OR	p

CST: Chi square test; MD: mean difference; MLR: multiple linear regression; OLR: ordinal logistic regression; OR: odds ratio; PR: Poisson regression

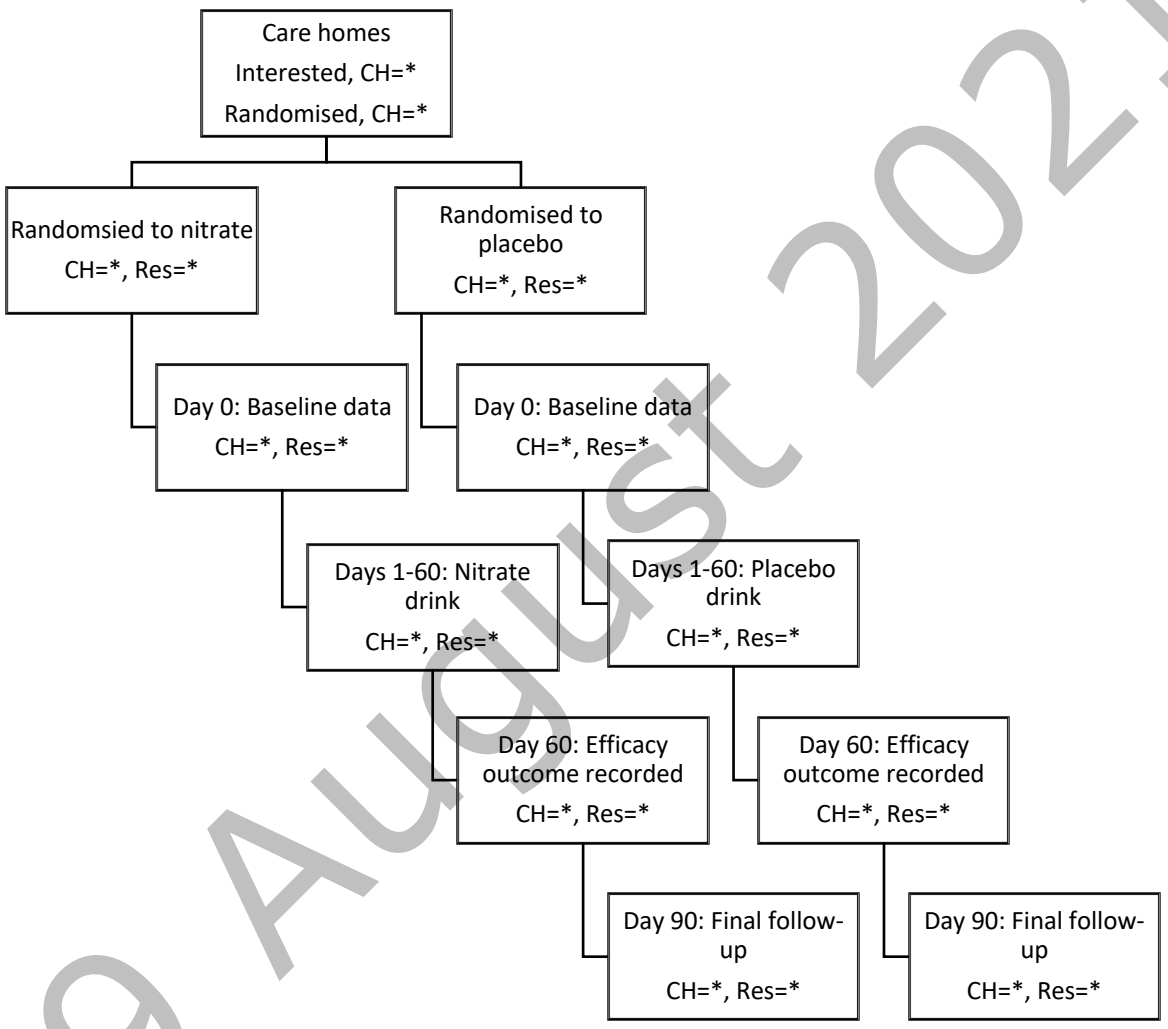
† Estimated from symptoms

We will also do an analysis that does not take account of clustering accepting that this is inappropriate but also the most sensitive way to see if there is any efficacy.

Table 5. Serious adverse events
Data are Number (%). Comparisons performed by Chi-Square test.

	Events			Participants		
Cause	**Nitrate**	**Placebo**	**P**	**Nitrate**	**Placebo**	**P**
N						
Type (%)						
Infection						
Heart Attack/MI or unstable angina						
Stroke						
Need of operation						
Fall						
Other						
If infection, type						
Respiratory (%)						
Gastro-intestinal (%)						
Urinary (%)						
Cutaneous (%)						
Other (%)						

Figure 1. Flow chart



CH: care homes; Res: residents

SUPPLEMENTARY DATA

Nitric oxide for preventing and reducing the severity of winter viral infections in care homes (BEET-Winter): a pilot trial

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The following supplement provides further information on the trial design and additional results.

PARTICIPATING CARE HOMES

Care homes who recruited and treated residents (number of residents)

Church farm Skylarks, West Bridgford, Nottinghamshire (11): Rachel Williams, Samantha McCormack.
Lynwood Court, Ascot, Berkshire (7): Maxine Freeman, Bonnie Trevellyan, Vikki Ribeiro.
Springbanks, Chesterfield, Derbyshire (8): Karen Busby, Laura Hill.
Wren Hall, Selston, Nottinghamshire (12): Anita Astle, Sophie Martin, Damian Mann.
Landermeads, Chilwell, Nottinghamshire (13): Ros Heath, Kimberley Borton, Katy Jackson, Helen Rain.

ELIGIBIITY CRITERIA

Care Home criteria
Inclusions
- Ideally CQC good or outstanding rating

Exclusions
- Staff "live" in care home
- Small homes <18

Resident criteria
Inclusions
- Age >=65
- Taking a normal / soft diet
- Willing to take treatment having taste-tested a beetroot shot

Exclusions:
- Participating in another randomised intervention trial
- No consent (resident, or family if resident lacks capacity)
- Using a thickener with food
- Feeding tube
- Using antiseptic mouthwash
- Currently has an infection requiring hospitalisation
- Identified by care home staff to be in last few days of life
- Short-term respite care
- Care home staff
- Takes beetroot juice daily

Supplementary Table 1. Design criteria in published cluster-randomised care home trials.

	Target	N	Homes	N/home	Active	Control	RRR	ARR	ICC	Alpha	1-ß
Infection											
Baldwin 2010 [20]	MRSA	480	24	20	15.3	17.0	10	1.7	0.01	5%	80%
Chami 2012 [21]	All	3,524	44	80	4.1	8.1		4.0	0.04	5%	90%
Mody 2015 [22]	UTI	418	12	35			23 S				
Gravenstein 2017 [23]	Influenza	75,917	823	92	3.4	3.9	11.2	0.5	0.35	5%	80%
Gravenstein 2018 [24]	Influenza	2,957	39	76	13.5	20.1	33.1	6.5	?	5%	?
Loizeau 2019 [25]	UTI/LRI	410	28	15				0.38	0.01	5%	90%
Arnold 2020 [26]	UTI	1,274	22	58	0.30	0.15	50	0.15	0.07	5%	80%
Teesing 2020 [27]	Hand hygiene		45	6	50	35		15	0.40	5%	80%
Others, selected											
OTCH 2015 [28]	OT	1042	228	4.6	5.5	5.3		0.2	0.37	5%	90%
Walker 2016 [29]	Falls	52	6	8.7	1.9	4.0			-	-	-
Logan 2021 [30]	Falls	1308	66	20	1.65pa	2.5pa	33%		0.10	5%	80%

ICC: intra-cluster correlation; LRI: lower respiratory tract infection; MRSA: methicillin-resistant *Staphylococcus aureus*; OT: occupational therapy; UTI: urinary tract infection

Supplementary Table 2. Care home recruitment

	No. of care homes
Intended	30
Expressions of interest	
Contracts signed	
Trained	
Recruited residents	
Randomised	
Commenced juice	
Completed juice	

Supplementary Table 3. Timelines for trial

Date	Event
28/07/20	Protocol, first draft
10/09/20	UPH submission
17/09/20	IRAS submission
21/09/20	UPH rejection
28/10/20	Research Ethics Committee meeting
09/11/20	Research Ethics Committee, provisional opinion
25/11/20	Research Ethics Committee, final approval (ID 288542)
	First contract with a care home
	First randomisation of a care home
	First care home received training on trial
	First care home completes baseline form
	First care home started nitrate supplementation
	Last care home started nitrate supplementation
	First care completed nitrate supplementation
	Last care home completed nitrate supplementation
	Last care home completed follow-up

Supplementary Table 4. Time to achieve milestones
Data are days, median [interquartile range]

	Contact	Contract	Testing	Random-isation	Training	Juice arrival	Consent	Baseline	Juice start	Day 14	Day 60	Day 90
Contact	X											
Contract		X										
Testing			X									
Randomisation				X								
Training					X							
Juice arrival						X						
Consent							X					
Baseline								X				
Juice start									X			
Day 14										X		
Day 60											X	
Day 90												X

Supplementary Table 5. Issues with trial

Issue	Implication	Remedy

Supplementary Table 6. Feasibility outcomes

Criteria	N (%)
Recruitment of 24 homes	
Number of taste tests	
Recruitment of 384 residents	
Recruited / taste tested	
Assessment of dietary nitrate	
Assessment of salivary/urinary nitrate	
Ability to measure ordinal outcome, >90%	
Mortality, all cause	
Hospitalisation, all cause [23]	
Infection in care home, needed healthcare input	
Infection in care home, no healthcare input	
No infection	
ICC (baseline assumption 0.01)	
Time to first infection [24]	

Supplementary Table 7. Feedback from care home managers.

Item	Median [IQR] / mean (SD)
Number of residents	
SARS-CoV-2 cases during Q1 2021	
Research in care homes	
Appropriate to do research in this vulnerable population	
Research in care homes is beneficial	
COVID-19 research in care homes is timely	
I am happy that my care home was involved in this research	
Important that my care home took part	
Taking part will be useful in marketing the trial	
Recruitment of residents	
Fewer than expected	
About what was expected	
More than expected	
Data collection	
Far too little collected	
Too little collected	
About right collected	
Too much collected	
Far too much collected	
Data entry by	
One staff member	
Two or more staff members	
Taste testing beetroot juice before enrolment was useful?	
Juice palatability	
Drank neat	
Diluted with orange juice	
Diluted with apple juice	
Drunk through a straw	
Starting juice	
All resident together	
Staggered start over several days	
Computers/tablets in care home	
Enough	
Trial-dedicated tablet would be useful	
Guestimate on randomisation	
Active (of total)	
Placebo (of total)	
Uncertain	
Free text comments	
Family	
Care home manager	